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Stereospecificity of alpha-chymotrypsin catalyzed hydrolysis

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STEREOSPECIFICITY OF α-CHYMOTRYPSIN CATALYZED HYDROLYSIS

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ABSTRACT

The study of enzymes and their catalytic actions has become an important aspect of modern biochemistry. Enzymes play an important part in the metabolism of living organisms because they catalyze most metabolic reactions. The absence or the loss of activity of a particular enzyme in the human metabolic cycle may cause serious disabilities such as in mental or physical development.

The stereospecificity of α-chymotrypsin catalysed hydrolysis of specific ester substrates has been investigated in this work. A series of diastereoisomeric phenylalanine ($\pm$) alkyl esters have been prepared and characterized by gas chromatography and mass spectrometry. A highly sensitive gas chromatographic technique was developed to follow the course of the enzyme hydrolysis quantitatively. Various methods of extracting significant information from the kinetic data were investigated and evaluated. The data obtained indicate that the structure and chirality of the alcohol moieties affect both the catalytic and binding constants of the substrates. The greatest difference between enantiomeric alcohol moieties are observed with esters derived from sterically crowded secondary alcohols. For racemic esters of the latter type, the stereospecificity of the alcohol site is such, that some resolution of racemic esters can be achieved via α-chymotrypsin catalysed hydrolysis on a preparative scale.

CONTENTS

- INTRODUCTION -

HISTORICAL BACKGROUND

Modern enzymology starts with Büchner's experiments on fermentation[1]; but tracing back the literature it seems that the first published paper on enzyme chemistry dates back to 1883 when Payen and Persoz[2] reported the existence of a biological substance which was capable of converting starch into sugar.

Büchner's experimental achievements on the fermentation processes totally disproved the controversial Liebig - Pasteur views on fermentation which was regarded by them to occur only in the presence of living cells and led the way for the isolation and identification of enzymes. It was not until 1926 that the protein nature of enzyme was confirmed.

During the early days of enzyme chemistry, researchers were concerned mainly with fermentation and digestive enzymes and very little work was done on intracellular enzymes. With the increasing awareness of the roles the enzymes play in biological

processes, workers oriented their research interests to different aspects of enzyme chemistry. These included studies on the metabolic processes concerned with the production and utilisation of energy and also metabolic disorders in mammals which are brought about by the lack of specific enzymes.

The purification of enzymes came into prominence with Sumner's work[3] on the crystallization of urease and since then many new techniques, viz. dialysis, electrophoresis etc. have been developed for the separation and purification of enzymes. The availability of pure and crystalline enzyme preparations have enabled workers to study enzyme kinetics and to carry out structural determinations on enzymes. A comprehensive study of enzyme kinetics and various kinetic measurements contribute to the understanding of the enzyme action and mechanism of enzyme catalysis.

The idea of enzyme specificity was introduced by E.Fischer[4] towards the end of the 19th century on the basis of his observation that enzymes are

active only on specific substrate structures.

The present concept of enzyme kinetics is largely a modified version of the classical researches of Henri [5] and Michaelis and Menten [6].

THEORY OF ENZYME KINETICS

The overall rate of an enzymic reaction (v) is usually determined by measuring the rate of disappearance of the substrate or the rate of formation of the product with time. Except for systems in which either the reaction products or the substrate itself causes inhibition, enzymic reactions are generally characterized by the following observations :

1. At constant initial substrate concentration $[S_0]$ the initial rate (v_0) is proportional to the enzyme concentration over a relatively wide range.

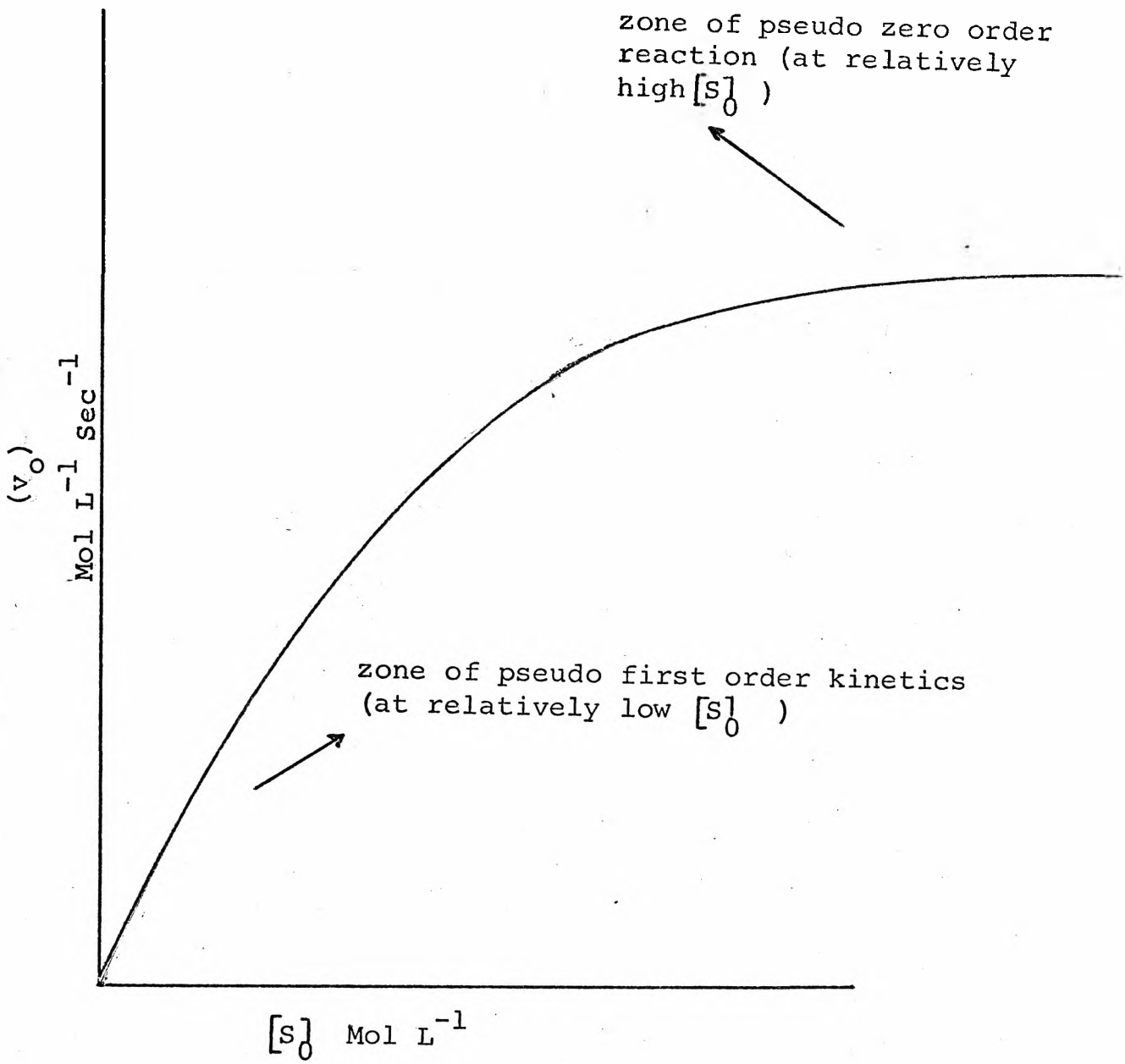
2. At constant initial enzyme concentration $[E_0]$ the initial rate of the reaction (v_0) increases with increasing initial substrate concentration.

The reactions of most enzymes which have been studied are characterized by a saturation phenomenon, i.e. when the initial reaction velocity (or the initial rate of the reaction) is plotted against the initial substrate concentration a curve of the type shown in the figure (1) is obtained.

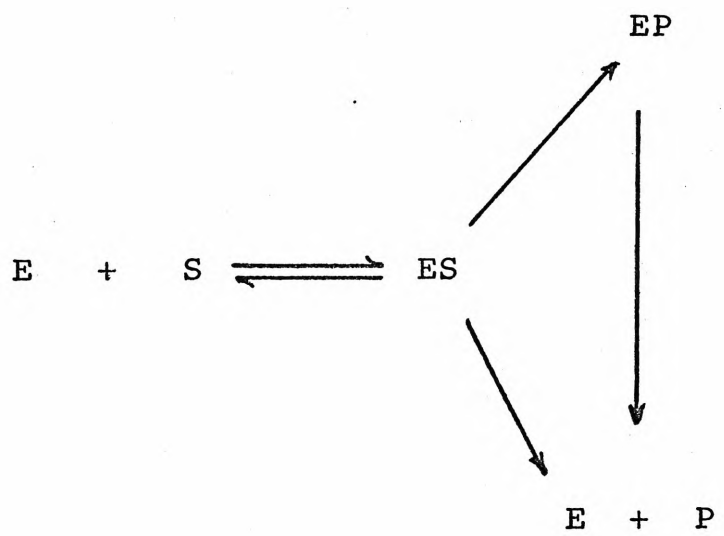
From the curve it is evident that at low $[S]_0$ values a linear relationship exists between (v_0) and $[S]_0$ but as $[S]_0$ increases the velocity eventually becomes independent of $[S]_0$.

NOTE: The terminology used in this chapter are used throughout the whole thesis. [ ] refer to the concentrations.

Figure (1). A plot of initial velocity of the reaction against the initial substrate concentration.



Most enzymic systems follow a definite reaction sequence which involves an initial reversible combination of the enzyme E with the substrate S to form an enzyme-substrate complex ES and which is followed by a transformation of the substrate bound to the enzyme surface to the enzyme-product complex EP. Finally this complex EP is decomposed to the product P and the free enzyme.



It is generally considered that the enzyme-substrate complex ES decomposes to the product and the free enzyme rather than the formation and the

subsquent decomposition of the enzyme-product complex EP since the formation and decomposition of EP is almost instantaneous, and hence its characterization in most cases has been impossible. The overall velocity of an enzymic reaction may be expressed by the following equation derived by Michaelis and Menten[6].

$$v = \frac{V_{max}\,[S]_0}{K_m + [S]_0} = \frac{V_{max}}{1 + K_m/[S]_0}$$

where V_{max} is the maximum velocity attainable and K_m is a constant known as the Michaelis-Menten constant.

Michaelis-Menten Theory:

Various kinetic studies on enzymic reactions suggest that the rate of the reaction is directly proportional to the substrate concentration over a wide range of low concentration but then tends to rise to a constant rate owing to a saturation phenomenon provided the enzyme concentration remains constant. Michaelis and Menten[6] suggested the concept of the enzyme-substrate complex and proposed a simple mathematical derivation to explain the above observation which bears their name and has since become an important and fundamental guideline in enzyme kinetics.

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P \quad \text{.....Eq (1)}$$

Assuming Eq (1) to be the reaction sequence for an enzyme substrate reaction where E, S, ES and P have their previous defined meaning and k_1, k_2, k_3 are the rate constants for the reactions indicated, then the rate of decomposition of the substrate with time is given by

$$-\frac{d[S]}{dt} = k_1 [E][S] - k_2 [ES] \quad \text{.....Eq (2)}$$

applying the steady state assumption the following relationship may be obtained.

$$-\frac{d[ES]}{dt} = k_1 [E][S] - k_2 [ES] - k_3 [ES] = 0 \quad \text{.....Eq (3)}$$

this is due to the fact that under steady state conditions the rate of formation and the rate of decomposition of ES are equal. Further if $[E_0]$ and $[S_0]$ are the initial concentrations of the enzyme and the substrate respectively then,

$$[E] = [E_0] - [ES] \quad \text{.....Eq (4)}$$

$$\text{and } [S] = [S_0] - [ES] \text{ ; which is} \simeq [S_0] \quad \text{.....(5)}$$

since, the substrate concentration used is invariably much larger than the enzyme concentration. Substituting the values for $[E]$ and $[S]$ in eq (3) the following equation may be obtained,

$$k_1 [S_0] ([E_0] - [ES]) - k_2 [ES] - k_3 [ES] = 0 \text{...eq(6)}$$

$$\text{or, } [ES] = \frac{k_1 [S_0] [E_0]}{k_1 [S_0] + k_2 + k_3} \quad \text{.....eq(7)}$$

Now, substituting the values of $[E]$ and $[S]$ in eq (2) and the value of $[ES]$ from eq (7) we get,

$$-\frac{d[S]}{dt} = k_1 [S_0] \left([E_0] - \frac{k_1 [S_0] [E_0]}{k_1 [S_0] + k_2 + k_3}\right) - \frac{k_1 k_2 [S_0] [E_0]}{k_1 [S_0] + k_2 + k_3}$$

.....Eq (8)

on simplification equation (9) may be obtained

$$-\frac{d[S]}{dt} = \frac{k_1 k_3 [E]_0 [S]_0}{k_1 [S]_0 + k_2 + k_3} \qquad \text{.....Eq (9)}$$

Further simplification can be done by assuming $k_1[S]_0 \gg (k_2 + k_3)$ at relatively high substrate concentration, therefore,

$$-\frac{d[S]}{dt} = \frac{k_1 k_3 [E]_0 [S]_0}{k_1 [S]_0} = k_3 [E]_0$$

$k_3[E]_0$ is a constant provided $[E]_0$ is a constant

or, $V_{max} = -\left(\frac{d[S]}{dt}\right)_{max} = k_3 [E]_0$

This accounts for the pseudo zero order reaction at relatively high substrate concentrations.

At relatively low $[S]_0$, $(k_2 + k_3) \gg k_1 [S]_0$

hence, $-\frac{d[S]}{dt} = \frac{k_1 k_3}{k_2 + k_3} \cdot [E]_0 [S]_0$ .....Eq(10)

which is a pseudo first order rate equation with the rate of the reaction depending on the initial substrate concentration $[S]_0$.

Thus for a series of reactions in which the initial enzyme concentration is constant but the initial substrate concentrations are varied

over controlled limits a rate profile such as figure (1) is obtained.

Eq (9) represents a form of Michaelis - Menten equation. The most common form of this equation, however, introduces a constant which is defined by the following expression $K_m = \frac{k_2 + k_3}{k_1}$

Dividing eq (9) by k_1, equation (11) can be obtained which is the most common form of the Michaelis-Menten equation.

$$-\frac{d[S]}{dt} = \frac{k_3 [E]_0 [S]_0}{[S]_0 + \frac{(k_2 + k_3)}{k_1}} = \frac{k_3 [E]_0 [S]_0}{[S]_0 + K_m}$$

.....Eq (11)

Significance and Evaluation of K_m and k_3:

Eq (11) contains two constants K_m and k_3 and on observation it appears that k_3 is the rate constant or the decomposition constant of the enzyme-substrate complex ES (Eq 1), while K_m is an approximation of the extent of dissociation of ES as defined by the expression $K_m = \frac{k_2 + k_3}{k_1}$. Hence an enzyme substrate combination with low K_m values would suggest a high degree of affinity of the enzyme towards the substrate but low k_3 values would signify slow rates of reaction or a decreasing catalytic action .

It has been observed that for most enzymic reactions a first order plot of Log $\frac{[S_0]}{[S]}$ against time t deviates from linearity towards the end of the reaction. This has been explained by the fact that in the Michaelis-Menten theory and its derivatives, the steady state assumption has been introduced to simplify the complex aspects of enzymic reactions and no allowance has been made for a number of side effects (eg. saturation of the enzyme at low substrate concentrations) that may take place during

the course of the reaction. The applicability of the Michaelis-Menten equation has been studied by various investigators[7-10] and it has been suggested that it can often best be used in an integrated form (equation 12)

$$k_3 [E]_0 = \frac{1}{t}\left[K_m \ln \frac{[S]_0}{[S]} + ([S]_0 - [S]) \right] \quad \text{.....Eq (12).}$$

There are a number of graphic methods for determining K_m. Lineweaver and Burke[11] first pointed out that eq (11) can be converted into a simple linear form

$$\frac{1}{v} = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]_0} + \frac{1}{V_{max}} \quad \text{.....Eq (13).}$$

Thus on plotting $1/v$ against $1/[S]_0$ a straight line with slope = K_m / V_{max} and intercept $1/V_{max}$ is obtained. A Lineweaver-Burke plot is particularly advantageous where the substrate concentrations are small and hence the reciprocal value is large. Eq (11) can be rearranged in two alternative linear forms[12,13] (equations 14,15).

$$[S]_0 / v = [S]_0 / V_{max} + K_m / V_{max} \quad \text{.....Eq (14)}$$

$$v = -\frac{v}{[S]_0} \cdot K_m + V_{max} \quad \text{.....Eq (15)}$$

Eq (14) gives a straight line plot of $[S]_0/v$ against $[S_0]$ with slope = $1/V_{max}$ and intercept K_m/V_{max}, and plotting v against $v/[S]_0$ from eq (15) yields a straight line with slope = $-K_m$. Each form offers certain advantages with particular range of data.

The integrated Michaelis-Menten equation eq(12) can also be used to determine K_m and V_{max}. Rearranging eq(12) into a linear form we have,

$$\frac{1}{t} \ln \frac{[S]_0}{[S]} = V_{max} / K_m - \frac{1}{K_m} \left(\frac{[S]_0 - [S]}{t} \right) \quad \text{.....Eq (16).}$$

plot of $\frac{1}{t} \ln \frac{[S]_0}{[S]}$ against $\left(\frac{[S]_0 - [S]}{t} \right)$ gives a straight line of slope $-\frac{1}{K_m}$.

The catalytic rate constant k_3 may be determined using eq(12) by plotting t against $K_m \ln \frac{[S]_0}{[S]} + ([S]_0 - [S])$ using the predetermined value of K_m.

GENERAL METHODS OF FOLLOWING ENZYME KINETICS

Two general methods of following the hydrolytic action of enzyme reactions are generally explored. The first involves the withdrawal of samples from the reaction medium while the other approach follows the enzymic reaction by measuring the change in some physical property of the reactant or the product, e.g. potential difference, pressure, optical properties, etc. The second method has the advantage that it does not disturb the reaction environment. In the first method (usually referred to as the sampling method) chemical analysis is done on either the substrate or one of the reaction products. At least three experimental measurements are necessary for the determination of the rate of the reaction; one at zero time (initial time), one after a suitable interval of time and the third at about twice this time which gives a check on the linearity of the curve over the time interval chosen. Generally care has to be taken that each sample is analysed as soon as it is withdrawn from the reaction mixture.

A general survey of the methods in use today for following the rate of enzymic reactions is briefly summarised below.

Spectrophotometric methods[14-17] are often used where the substrate or one of the products of the enzymic reaction has a chromophoric group. This method is technically simple and sampling is not required. It is important that the spectrophotometer cells, in which the enzyme reactions are being carried out are thermostatically controlled inside the cell compartment of the instrument. Because the substrate and the product rarely have the same spectrum the enzymc reaction can usually be followed by choosing a suitable wavelength at which there is a considerable change in the absorption as the reaction proceeds.

With the development of instruments such as the Radiometer "pH Stat" which maintains a constant pH by the addition of acid or alkali, methods which are based on pH changes have become quite popular for enzyme studies. This method can also

be adapted for studying oxidising enzymes and in these cases a redox potential instead of pH is measured. A detailed review describing studies using the Radiometer pH Stat has been published[18]. Polarimetric methods[19] are also suitable for the study of enzymic reactions whenever an enzyme acts stereospecifically on a substrate. In these cases the enzyme acts on only one of the stereoisomers and the rate of the reaction can be followed by measuring the change in the optical rotation against time. This method can also be used where the substrate is racemic but an optically active product is formed, or where the substrate and the product are both optically active but differ considerably in either the sign or the degree of specific rotation. Other methods which have often been used in enzyme studies are colorimetry[20,21], manometry[22], and chromatography. Each method is in some cases highly specific, accurate and convenient; for example, manometry has been used in the study of oxidases or carboxylases.

The application of chromatography to the study of enzyme kinetics is a rather recent development. In this case samples have to be withdrawn from the reaction mixture at suitable interval of time and analysed either by thin layer (T.L.C.), paper or gas liquid chromatography (G.L.C.). Both paper and T.L.C. are not ideally suitable for kinetic studies because they are only semi-quantitative, but G.L.C. is potentially more suitable for this purpose. The first application of G.L.C. by James and Martin[23] was aimed at biochemical applications and since then many workers have used the method for a number of biological studies[24-30]. In G.L.C. the sample is injected into a preheated system where it is vapourised, and then carried by a stream of inert gas, into a column packed with a high boiling liquid phase supported on an inert solid support. The sample components are then separated by partition between the gaseous and the liquid phases.

Compounds which are not volatile enough for G.L.C. analysis must first be converted into suitable volatile derivatives which must be stable at the temperature required for G.L.C.

Though G.L.C. is a comparatively new method for enzymic kinetic studies, it is probably one of the best method to study a stereospecific enzymic reaction. By choosing a suitable G.L.C. column it is possible to resolve a diastereoisomeric substrate into its two individual components and hence the steric course of the reaction can be studied.

Finally it should be noted that an allowance has to be made for any non enzymic hydrolysis that might occur during the experimental run. This is always done by running a blank experiment without the enzyme but in which all other experimental conditions have been duplicated.

MECHANISM OF ENZYME ACTION

Since enzymes are proteins they are more sensitive to temperature and pH conditions than chemical catalysts. Although enzyme catalysis is chemical in nature there are some significant differences between enzymic and chemically catalyzed reactions.

1. The rate of an enzyme catalyzed reaction is generally much faster than the rate of a similar reaction catalyzed by a chemical agent.
2. The catalytic action of an enzyme is usually more selective and it is only active at a physiological temperature while chemically catalyzed reactions may require higher temperatures.

The actual mechanism involved in enzymic catalysis is still largely unknown, but it seems clear that the initial step in an enzyme catalyzed reaction involves the binding of the substrate to the enzyme surface. Such a substrate attachment suggests that the binding sites of the enzyme might orient themselves in a specific way to make such attachment possible. This concept of an enzyme

active site involves consideration of the orientation effect as well as the proximity effect. This has been investigated in some detail by Koshland[31] who has concluded that:

1. The rate of adsorption and desorption of the substrates and the products from the enzyme surface appear to be infinite.

2. The concentration of the enzyme represents the concentration of the active sites.

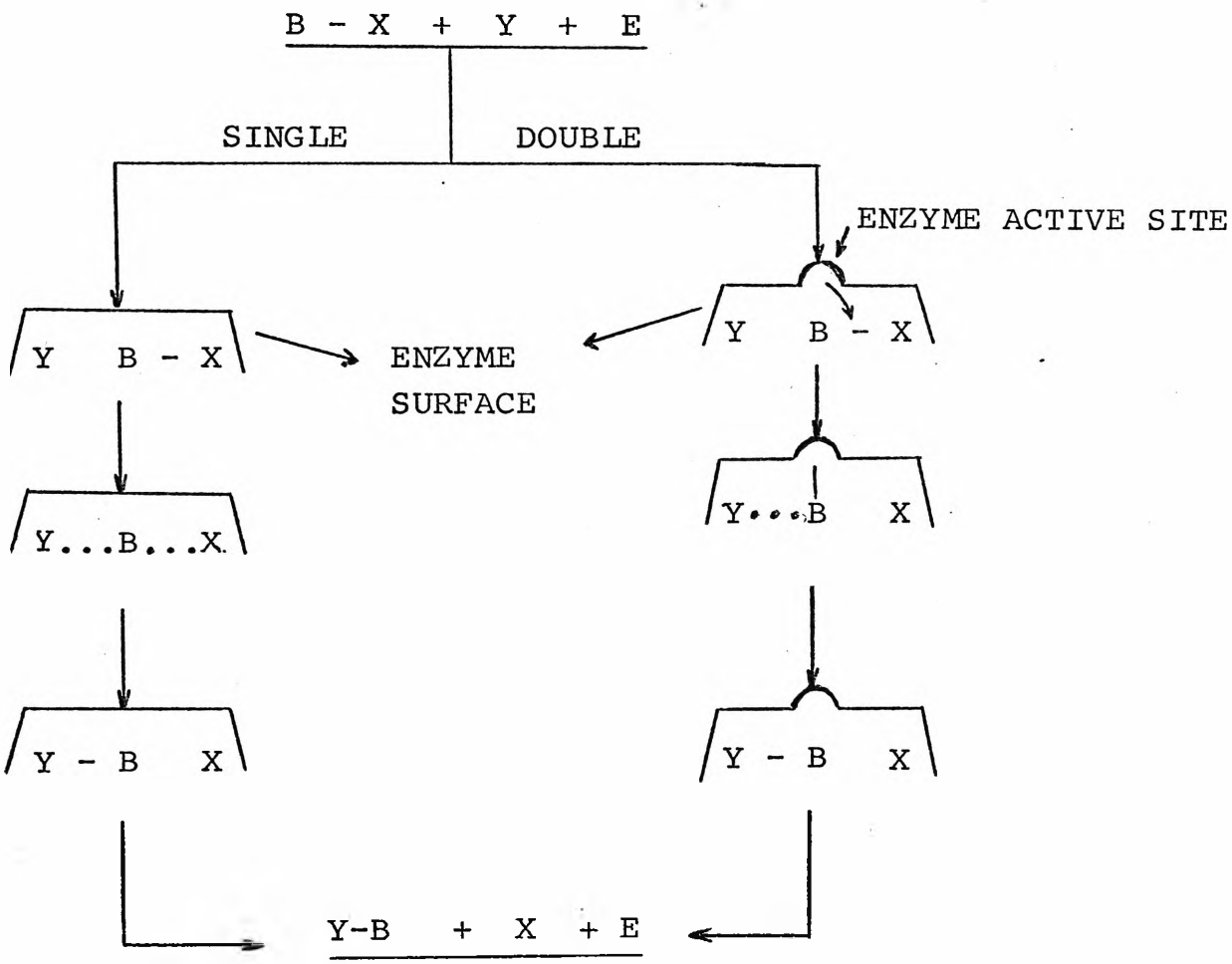
3. The concentration of the reactants on the enzyme surface depend on the concentration of the active sites on the enzyme surface.

Koshland[31] also proposed that enzymes may catalyze a reaction by either a single or a double displacement mechanisms. In the single displacement mechanism the enzyme acts as a surface on which the substrate molecules may be held in contact with each other, by hydrogen bonding. The crowding of the substrate molecules on the enzyme surface may increase their collision frequency which might result in decomposition to the product and the free enzyme; or some substrate molecules

might be trapped in some of the active sites on the enzyme surface which could then lead to decomposition under a strained condition to a product.

In the double displacement mechanism the enzyme initially participates directly in the catalytic process by the formation of an enzyme-substrate complex which is then attacked by an acceptor group. (figure 2).

Figure 2. Displacement Mechanisms of Koshland



B-X ——— DONOR SUBSTRATE

Y _____ ACCEPTOR

Koshland's [31] theory of a double displacement mechanism for enzyme reactions suggests the existence of an intermediate (the enzyme-substrate complex) and this has now been verified and characterized in a number of instances.

The high catalytic efficiency and remarkable specificity of many enzymes have led to the belief that in an enzyme molecule there are always a number of active functional groups to which the catalytic action of the enzyme may be ascribed to. This has given rise to the concept of the "active sites" within an enzyme molecule. Studies with α-chymotrypsin have provided some clues, regarding the mechanism of the action of this enzyme. Thus, α-chymotrypsin is an acyl group transfer enzyme, and it transfers the acyl groups from a number of donors viz. an N-acyl-aminoacid or an amino acid ester, or amino acid amide etc. to a number of acceptors such as water, alcohols, or amines etc.

The following experimental observations have supported the concept of an acyl-enzyme intermediate formation, for α-chymotrypsin and other related proteolytic enzymes.

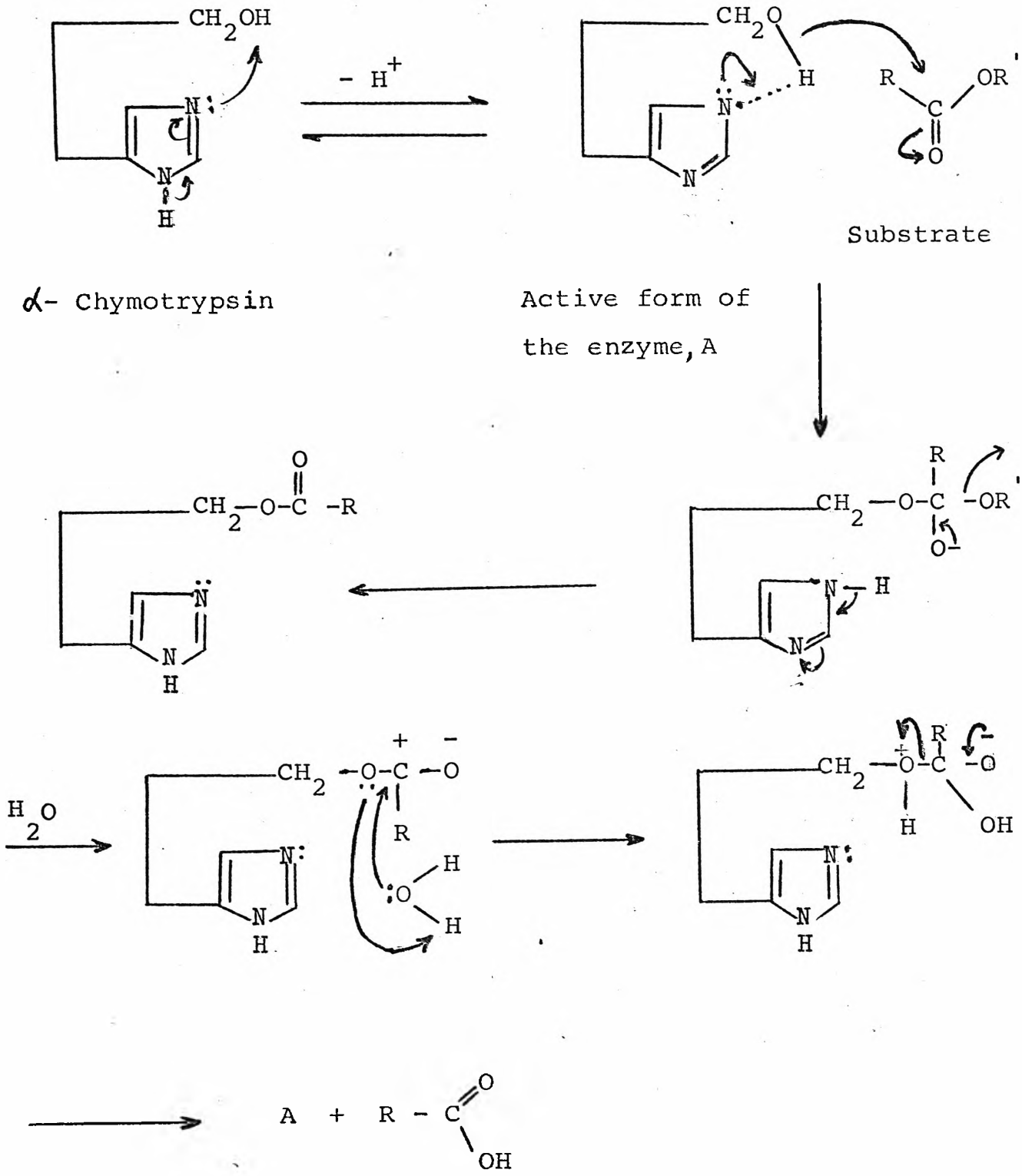
1. The acyl-enzyme intermediate has been demonstrated spectrophotometrically in many α-chymotrypsin catalyzed reactions.[32-35]

2. The reaction kinetics of an α-chymotrypsin catalyzed reaction in the presence of an added nucleophile like methanol, ethanol has indicated the presence of a competitive reaction between the nucleophile and the acyl-enzyme intermediate.

3. Chemical and enzymic degradations of several acyl-chymotrypsin intermediates have revealed that the acyl group is attached to the hydroxyl group of the serine residue in position 195 of the enzyme[36].

The enzymic hydrolysis of α-chymotrypsin can be explained by the following mechanism, figure (3).

Figure (3). Mechanism of α-chymotrypsin reaction; formation of enzyme-substrate complex

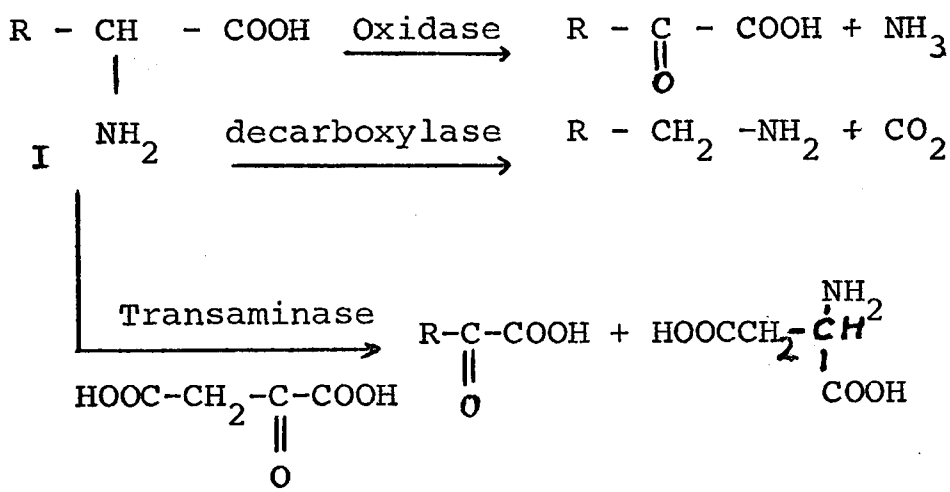


The scheme shows that the serine in position (195) and the histidine residue in position (57) are responsible for the catalytic action of the enzyme. The histidine residue in position (57) can act as a proton acceptor. The reaction first proceeds by the binding of the substrate through its carboxyl function to the serine 195 residue of the enzyme to form an O-acyl enzyme derivative. The ester bond is then hydrolyzed by the nucleophile present (water or alcohol etc.) and the enzyme returns to its original reactive form A (figure 3) which undergoes this process again.

The formation of an acyl-enzyme intermediate have also been postulated for reactions catalyzed by cholinesterase[37], trypsin[38,39], and many other proteolytic enzymes.[40]

ENZYME SPECIFICITY

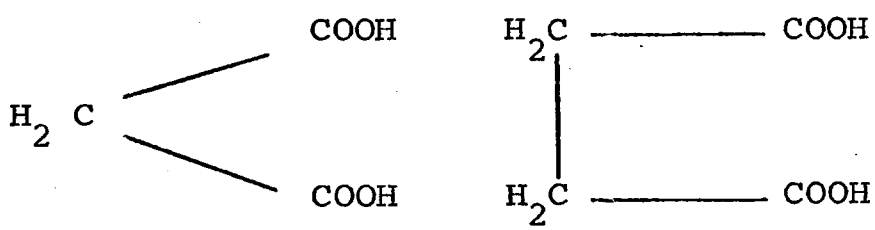
One of the most characteristic features of an enzyme is its ability to exhibit a certain degree of selectivity in its reactions. The degree, type and the nature of this selectivity varies with different enzymes and this can be demonstrated by the following enzymic reactions.



In these reactions, the aminoacid I undergoes three different types of reactions which are catalyzed by three different enzymes. The enzyme oxidase oxidatively deaminates the aminoacid I while decarboxylation of I is achieved by decarboxylase. Transamination of the aminoacid I requires the enzyme transaminase which helps in the

interchange of the functional groups between the amino group of I and the keto group of oxaloacetic acid.

In most enzymic reactions a substrate molecule is bound to the enzyme surface before catalysis can occur. Because the binding of the enzyme to the substrates depends on the molecular structure and configuration of both enzyme and the substrate, enzymes usually cannot act on a large variety of substrates. For example, α and β-galactosidase can only hydrolyse α and β-galactose while succinate dehydrogenase can only dehydrogenate succinic acid but not malonic acid which are structurally similar.



Malonic acid Succinic acid

Similarly carboxypeptidase requires a substrate which has a free carboxyl group, and amino acid amides and esters are not attacked. Leucine aminopeptidase acts only on an amino acid substrate, which contains a free amino group and α-chymotrypsin can only be used efficiently for the hydrolysis of a "bound" aromatic aminoacid e.g. phenylalanine, tyrosine or tryptophane.

In addition to being highly specific, most enzymes show a remarkable stereospecificity towards certain substrates. There are many examples where an enzyme acts on only one of the isomers of a racemic substrate.

For example,

$$\text{L - amino acid} \xrightarrow[[O]]{\text{L - amino oxidase}} \alpha\text{-Keto acid} + H_2O_2 + NH_3$$

$$\text{D - amino acid} \xrightarrow[[O]]{\text{D - amino oxidase}} \alpha\text{-Keto acid} + H_2O_2 + NH_3$$

Another interesting aspect of enzyme specificity is the fact that many enzymes are capable of distinguishing chemically identical groups within a substrate molecule.

Two examples are cited below;

The first involves the glycerokinase reaction (figure 4). The product of the reaction is exclusively L-α-phosphoglycerol. This reaction indicates that the enzyme treats the chemically identical hydroxymethyl groups of glycerol in an asymmetric fashion, since if this was not the case then a mixture of L-α and D-α- physphoglycarol would have been formed.

The second example relates to the behaviour of the enzyme aconitase which metabolizes citric acid. Citric acid labelled in a primary carboxyl group, prepared from ^{14}C labelled oxaloacetic acid is converted enzymatically to 2-ketoglutaric acid. Chemical degradation of the product reveals that ^{14}C labelling occurs only at the carboxyl group adjacent to the carbonyl function indicating that aconitase distinguishes between the chemically identical carboxymethyl groups of citric acid (figure 5). The first product of the reaction isocitric acid is formed as a racemic mixture with the ^{14}C labelling on the carboxyl group attached to the terminal -CH(OH) group.

Figure (4) The conversion of glycerol to L-α-phospho-glycerol by glycerol kinase

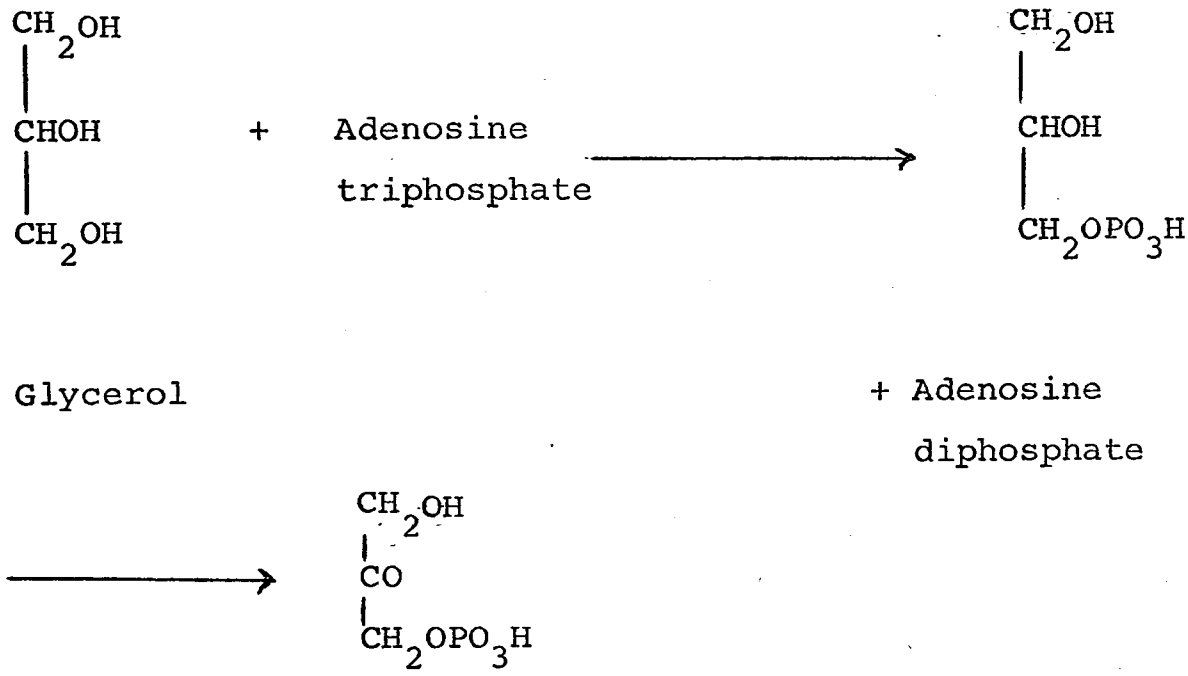
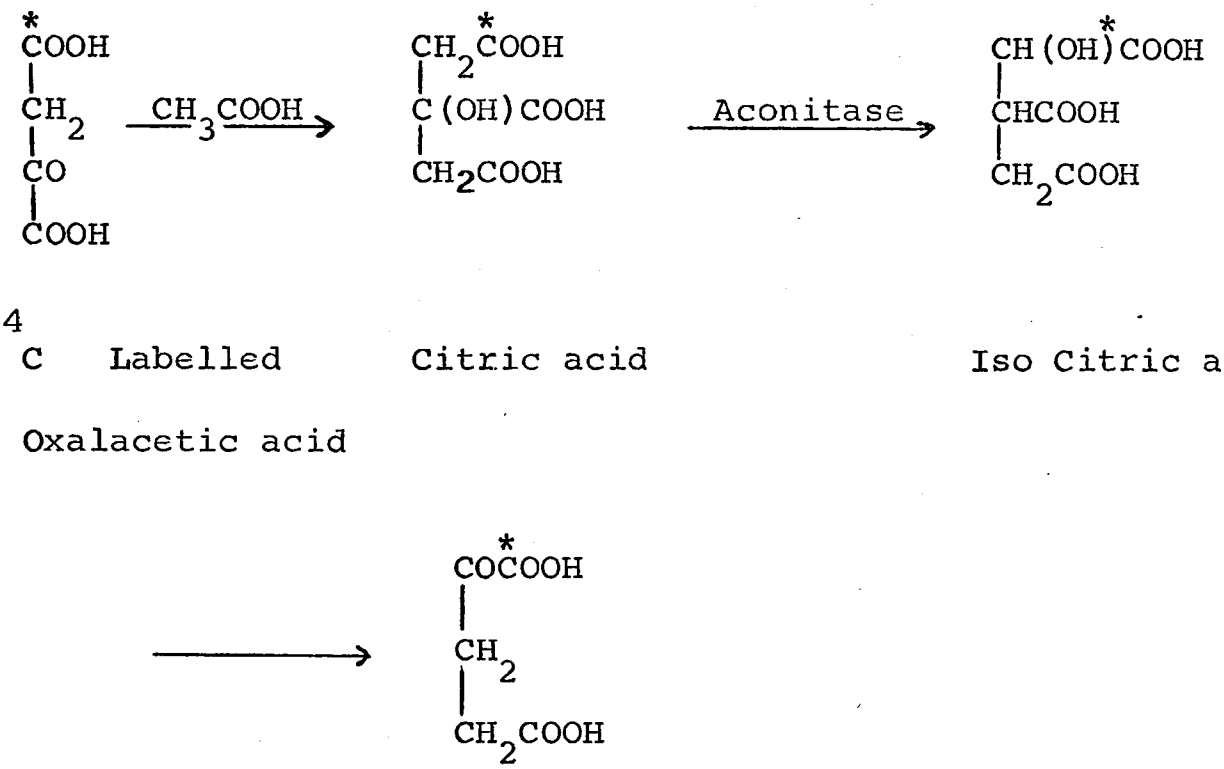


Figure (5) Conversion of citric acid to 2-ketoglutaric acid by aconitase .



^{14}C Labelled Oxalacetic acid

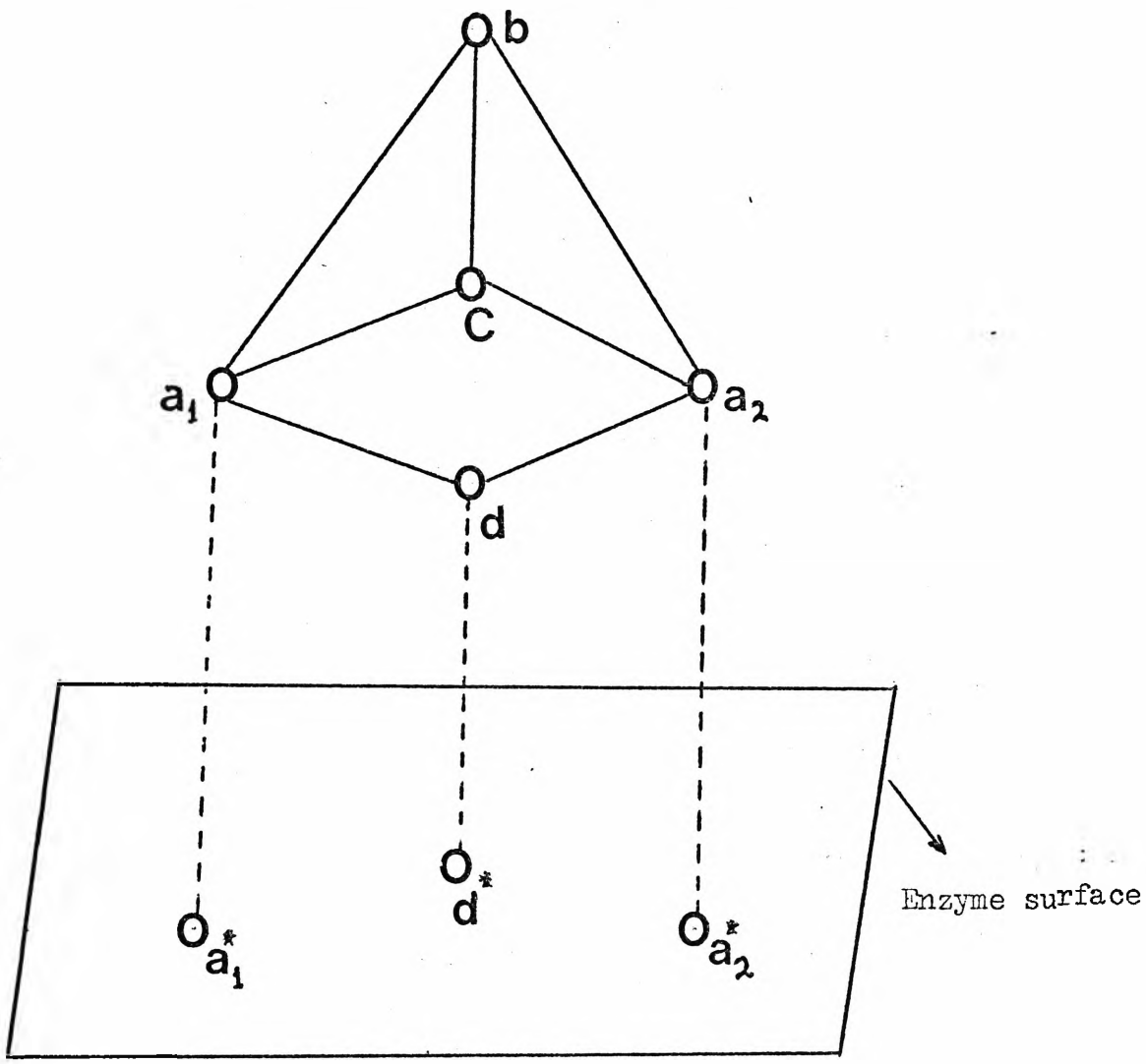
Citric acid

Iso Citric acid

2-Keto Glutaric Acid

Ogston[41,42] has pointed out that the enzymatic differentiation of chemically identical groups can be rationalized on the basis of a three point attachment of substrate to the enzyme surface. Such a situation is depicted schematically in the figure(b). The groups a_1, a_2 and d of the substrate molecule are attached to points a_1^*, a_2^* and d^* of the enzyme active site and the spatial orientation of the points of attachment of the substrate are such that the enzyme-substrate combination achieves the best structurally fit positions.

Figure (6). Model of Three Point Attachment of the Substrate to the Enzyme Surface



groups a_1 and a_2 are chemically identical

-DISCUSSIONS AND RESULTS-

Mass Spectrometry of Phenylalanine alkyl Esters

The present work was aimed at the investigation of the stereospecific action of α-chymotrypsin on L-phenylalanine ($\pm$) alkyl esters using G.L.C. as a means of following the reaction.

A number of substrates were synthesised by published esterification methods[43] in which phenylalanine the alcohol and p-toluenesulphonic acid were refluxed in a mixture of benzene and toluene. The substrates were obtained as crystalline phenylalanine alkyl ester tosylates, from which the free phenylalanine alkyl esters could be liberated by treatment with base.

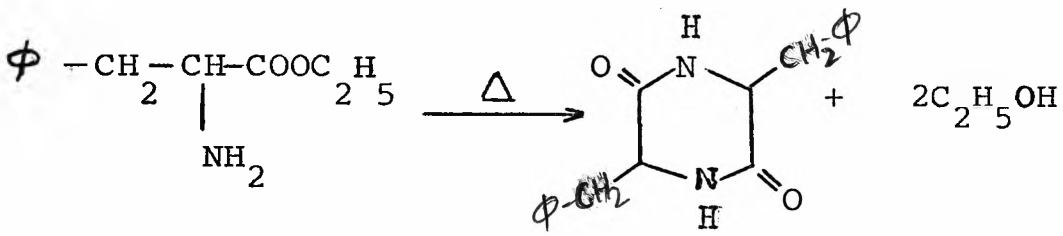
The chemical purification of the diastereoisomeric substrates proved particularly difficult as crystallization, distillation or sublimation of the crude tosylates of the L-phenylalanine alkyl esters invariably resulted in partial separation of the diastereoisomers. Though the substrates were prepared in excess of (98%) chemical purity (G.L.C. analysis), unfortunately an analytically pure sample could not be obtained without a partial resolution of the diastereoisomeric mixtures. In view of this

difficulty we relied on G.L.C. for the analysis of the diastereoisomeric mixture and on M.S. for the structural identification of the substrates. The interpretation of the mass spectrometric fragmentation patterns of the phenylalanine alkyl esters proved to be quite straight forward as the M.S. of ethyl and methyl esters of L-phenylalanine had been investigated previously[44,45]. L-phenylalanine ethyl ester on electron bombardment gives a number of characteristic ion fragments of which the amine fragment (m/e 120) and the ester fragment (m/e 102) are diagnostic. Both the fragments are stabilized by the presence of the nitrogen lone pair electrons and the structure of the amino acid side chain can be deduced from their m/e values.

It has been observed[46] that all α-aminoacid esters undergo a dimerization reaction to form diketopiperazines when heated in the ion source of the mass spectrometer (Reaction 1). This results in a lower abundance of the amine and the ester fragments and hence the sensitivity of the method

is considerably reduced.

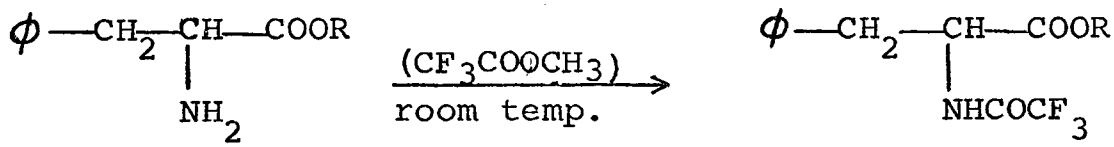
Reaction 1



Diketopiperazine

It was therefore desirable to chemically modify the phenylalanine alkyl esters before the mass spectrometric analysis. This was done simply by derivatizing the phenylalanine alkyl ester substrates with trifluoroaceticanhydride. Alternatively, the trifluoroacetyl group could be introduced by treating the esters with methyl trifluoroacetate in the presence of a base (Reaction 2).

Reaction 2



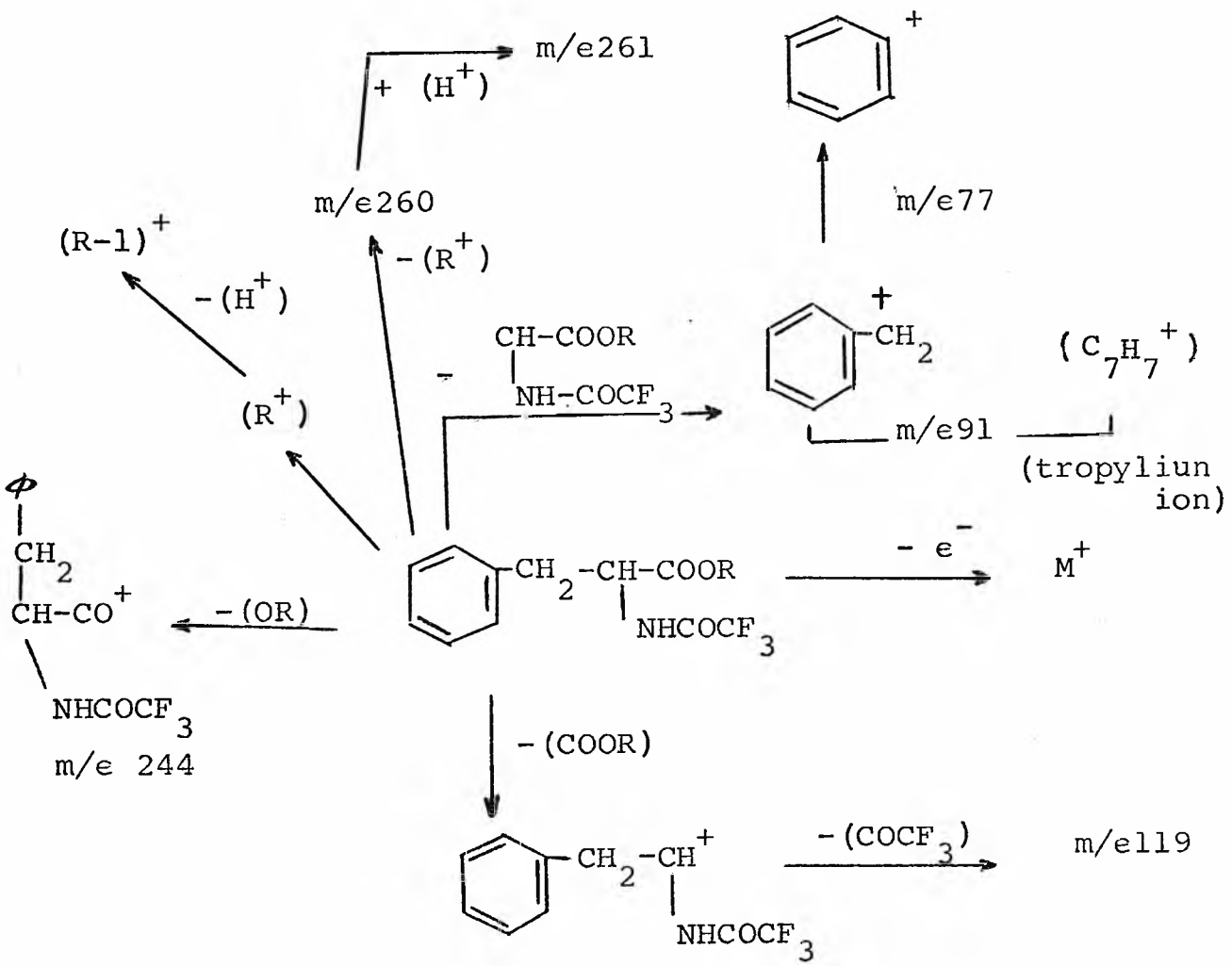
N-TFA-phenylalanine alkyl ester

The M.S. of N-TFA phenylalanine alkyl esters in general followed a similar fragmentation pattern to those of the free esters but the formation of diketopiperazines was avoided. The amine fragment (m/e 216) was the base peak in all N-TFA phenylalanine alkyl esters studied, although the ion intensities varied depending on the alkyl ester function. In addition peaks at m/e 91 ($C_7 H_7^+$, tropylium ion),

m/e 77 ($C_6H^+_5$, phenylium ion) and m/e 69 (CF^+_3) were present in the M.S. of all the N-TFA phenylalanine alkyl ester substrates. The formation of the molecular ion (M^+) was observed in most cases although its abundance was often quite low (0.1%). The presence of a substituent in the aromatic ring of the phenylalanine alkyl ester does not alter the mass fragmentation pattern . For example, N-TFA derivatives of p-fluoro and p-nitro DL-phenylalanine gave amine fragments at m/e 234 and at m/e 261 which were as expected 18 and 45 a.m.u. higher than the corresponding unsubstituted phenylalanine compounds. The molecular ion was obtained in all cases and the abundance of the molecular ion for p-nitro DL-phenylalanine compounds were approximately 1%.

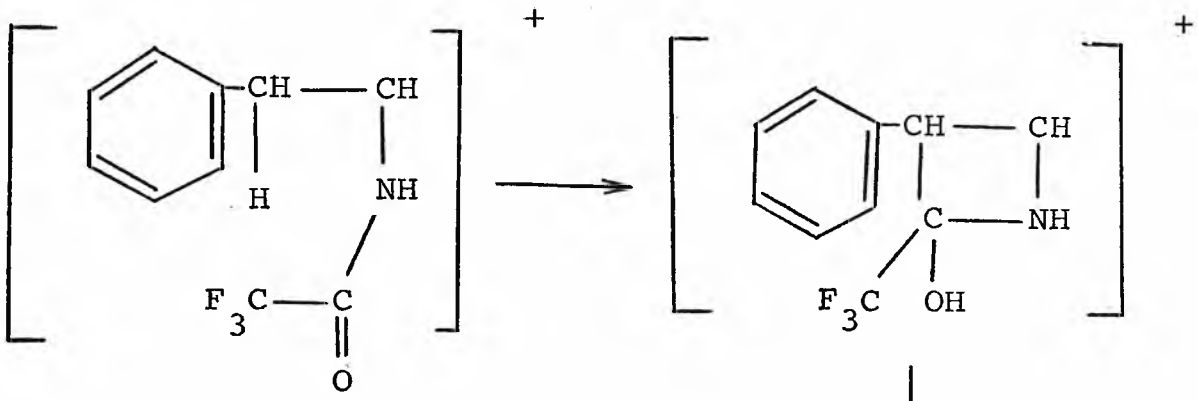
A general scheme of the mass spectral fragmentation of N-TFA phenylalanine alkyl ester is given in figure (7) and the characteristic M.S. fragment ions for the various substrates are summarized in table (1).

Figure (7). A General Scheme of Mass Fragmentation of N-TFA-phenylalanine alkyl esters

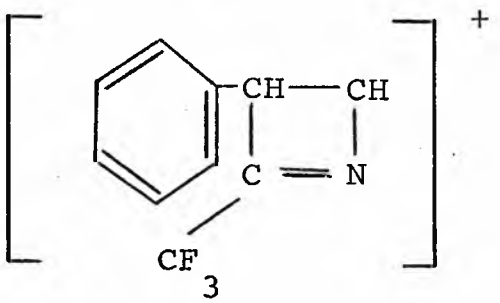


(m/e216, amine fragment)

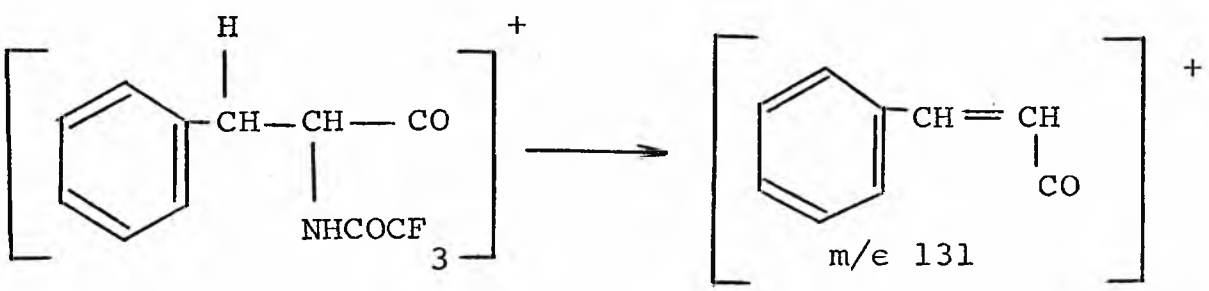
.....(continued)



Amine fragment m/e 216



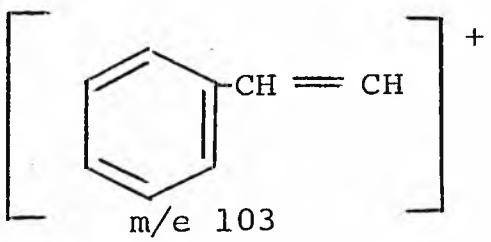
m/e 198



m/e 244

m/e 131

-(CO)



m/e 103

Table (1). Mass-spectrometry of N-TFA phenylalanine alkyl esters at 200°C and 70e.v energy

		M^+	R^+	$(R-1)^+$	$(M-COOR)^+$	$(M-COCF_3)$	$(C_7H_7)^+$	OTHERS
1	methyl	275	-	-	216	178	91	-
2	ethyl	289	-	-	216	192	91	-
3	n-propyl	303	43	-	216	206	91	-
4	n-butyl	317	57	56	216	220	91	-
5	2-butyl	317	57	56	216	220	91	-
6	2-methyl-1-butyl	331	71	70	216	234	91	$^+CH\langle^{CH_3}_{C_2H_5}$
7	3-methyl-2-butyl	331	71	70	216	234	91	$^+CH(CH_3)_2$
8	3,3-dimethyl-2-butyl	345	85	84	216	248	91	$C^+(CH_3)_3$
9	amyl	331	71	70	216	234	91	-
10	3-methyl-2-pentyl	345	85	84	216	248	91	-
11	4-methyl-2-pentyl	345	85	84	216	248	91	-
12	2-methyl-3-pentyl	345	85	84	216	248	91	$^+CH(CH_3)_2$
13	cyclohexyl	343	83	82	216	-	91	-
14	2-methyl-cyclohexyl	357	97	96	216	-	91	-
15	n-octyl	373	113	112	216	276	91	-
16	2-octyl	-	113	-	216	276	91	-
17	menthyl	-	139	138	216	302	91	$(C_7H_{11})^+$
18	1-cyclohexyl-ethyl	371	111	110	216	-	-	$(C_6H_{11})^+$

Gas Liquid Chromatography of Phenylalanine alkyl Esters

The use of gas liquid chromatography for the resolution of diastereoisomeric compounds is a relatively new method and it is only in the last decade that the scope and usefulness of this analytical procedure has been evaluated. Weygand et.al.[47] have applied this method for the resolution of diastereoisomeric dipeptide derivatives and Halpern et.al.[48] have used this technique to study a number of biochemical problems.

The gas chromatographic resolution of a mixture of optical isomers can be achieved by two methods. The first involves the use of a G.L.C. column packed with an optically active stationary phase[49], the second involves the conversion of the enantiomers to diastereoisomers prior to G.L.C. analysis. The applicability of the first method to the separation of aromatic amino acids has not been described so far in great detail. So our approach to G.L.C. resolution of optical isomers was based on the use of inactive stationary phases. Asymmetric alcohols have been resolved[50]

by the conversion into a mixture of diastereoisomeric esters of an optically pure acid, and capillary column with both polar and non polar phases have been used. The extent of successful resolution depended mainly on the stationary phase and the nature of the alcohol, and it has been suggested in the literature[51] that the resolution factor (the ratio of the retention time of the two diastereoisomers) increases with the increase in the chain length of the alcohol. It has also been reported[52] that the use of a strongly polar stationary phase improves the resolution. Westley et al[53] have resolved a number of secondary alcohols (C_4 - C_7) via the N-TFA phenylalanine esters.

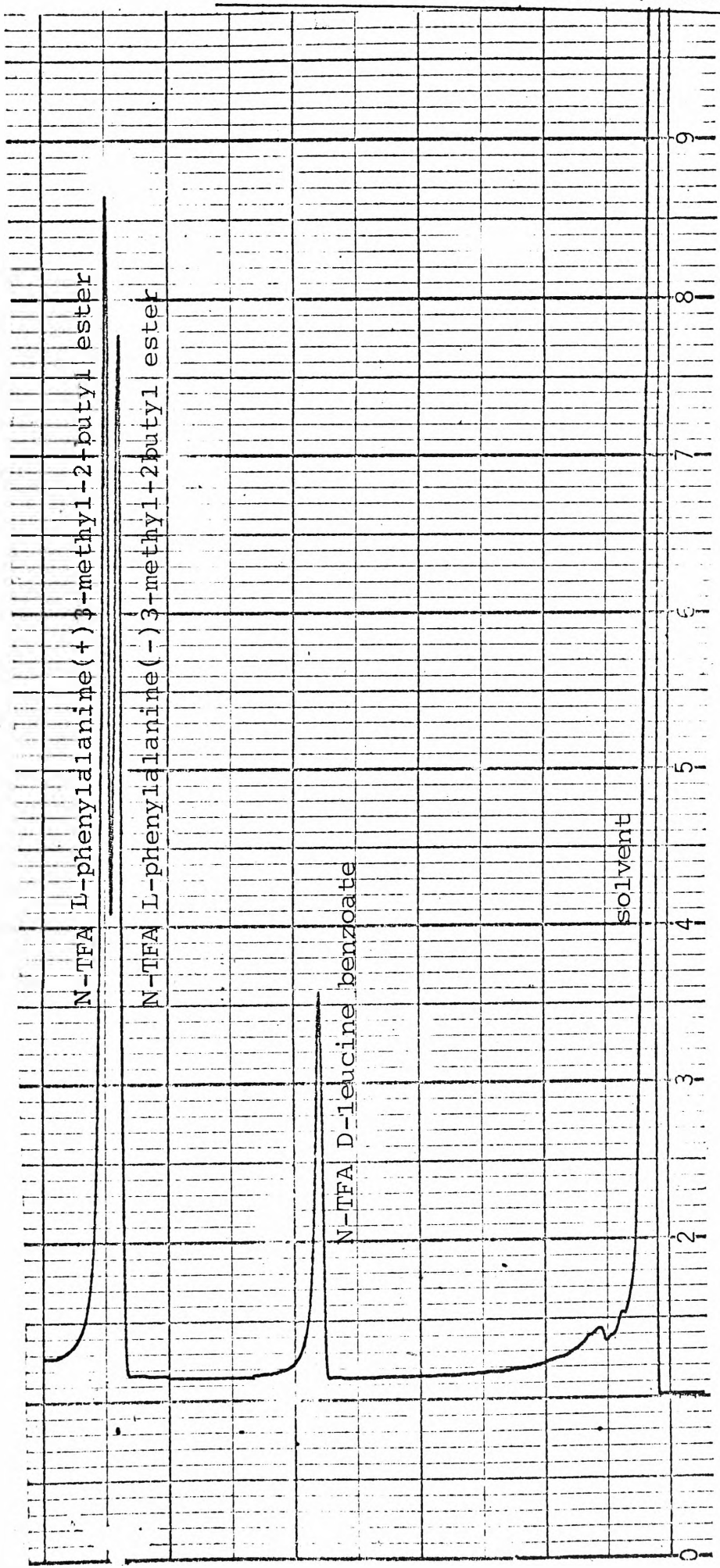
Since phenylalanine alkyl esters are not stable at elevated temperatures it is not possible to analyze them quantitatively by G.L.C. Hence it was necessary to convert them into derivatives suitable for G.L.C. analysis. N-~~trifluoro-acetyl~~ TFA derivatives were chosen because they are readily prep-

ared under mild conditions and the derivatization reaction is quantitative (can be followed by T.L.C. by the disappearance of the ninhydrin positive spot). They are more volatile and stable at elevated temperatures than their parent amino acid esters and can be stored for a considerable length of time without structural alteration.

A number of columns were tested to determine the suitability of the G.L.C. analysis of N-TFA phenylalanine (±) alkyl esters and N-TFA phenylalanine optically pure and inactive alkyl esters.

After a series of experiments with various G.L.C. columns it was found that a highly polar column (0.7/0.3% w/w DEGS/EGS-X on Chromosorb-W) was suitable for the resolution of the N-TFA-L-phenylalanine (±) alkyl esters. Because these liquid phase columns tend to bleed rather heavily at temperatures $> 200^{o}C$, a suitable analysis temperature was determined by a strict temperature programme ($4^{o}C$/min) and carrier gas flow. Figure (8) gives a typical separation for N-TFA-L-phenylalanine (±) 3-methyl-2-butyl ester, the resolution

Figure (8) G.L.C. Separation of L-phenylalanine (±) 3-Methyl-2-butyl ester (N-TFA derivative)



factor obtained was 1.07. Table (2) gives the retention times and the separation temperatures of a series of N-TFA-L-phenylalanine (±) alkyl esters which were used for kinetic studies.

Halpern et al[54] have also published the resolution data for a number of 2-and 3-alkanols via the N-TFA phenylalanine esters. The authors[54] have used a strongly polar stationary phase. Table (3) summarizes the resolution factors of a number of diastereoisomeric phenylalanine esters obtained in this study and some results obtained by Halpern et.al[54].

A general observation of the published data (a review on the resolution of optical isomers by gas chromatography has been published by E.Gil-Av and D.Nurok[55]) on the separation of different types of diastereoisomeric compounds reveal that resolution is more effective on polar columns than on non-polar columns. This observation leads to the conclusion that the degree of diastereoisomeric resolution must be associated with the polarity

Table (2)

* G.L.C. analysis of N-TFA L-phenylalanine(±)alkyl esters

(±)R	RETENTION TIME (min) L(+)	RETENTION TIME (min) L(-)	SEPARATION TEMP. °C	RESOLUTION FACTOR	$\Delta(\Delta G^{\circ})$+ Calories Mol^{-1}
2-methyl-1-butyl x	7.70	7.70	-	-	-
2-butyl	8.47	7.92	183	1.07	-61.4
3-methyl-2-butyl	9.25	8.62	186	1.07	-61.8
3-methyl-2-pentyl	10.75	10.08	191	1.07	-62.5
4-methyl-2-pentyl	9.25	8.85	186	1.05	-44.6
2-methyl-3-pentyl	10.33	9.70	189	1.06	-53.5
3,3'-dimethyl-2-butyl	10.75	9.92	190	1.08	-70.8
2-Methyl cyclohexyl.	15.84	15.20	200 (Isotherm)	1.04	-36.9

* Column: 6'x1/8" stainless steel column packed with 0.7/0.3 w/w% DEGS/EGGS-X over D.M.C.S. treated chromosorb-W
Temperature programing was 150°-200° C at 4°C/min.
N_2 flow 25 ml/min

x Diastereoisomers not resolved on this column.

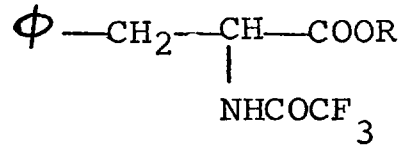
+ Calculated from equation

$\Delta(\Delta G^{\circ}) = - RT \ln$ (resolution factor)

where the resolution factor is the ratio of retention times of the two diastercoisomers (cf W.Parr and P.V.Howard, Analytical Chemistry 45, 711, 1973); and R=1.99 Calories/mole/°K

Table (3)

A Comparison of G.L.C. data for N-TFA Phenylalanine alkyl esters

GENERAL STRUCTURE 

R	RESOLUTION FACTOR *	RESOLUTION FACTOR **	$\Delta(\Delta G^{O})$ *	$\Delta(\Delta G^{O})$ **
2-butyl X	1.07	1.04	- 61.4	- 32.1
3-methyl-2-butyl X	1.07	1.09	- 61.8	- 73.4
3-methyl-2-pentyl X	1.07	1.08	- 62.5	- 65.5
2-methyl-3-pentyl	1.06	1.03	- 53.5	- 25.2
4-methyl-2-pentyl	1.05	1.03	- 44.6	- 25.2
3,3'-dimethyl-2-pentyl	1.08	1.15	- 70.8	-119.0
2-methyl cyclohexyl	1.04	1.09	- 36.9	- 75.9

G.L.C. condition :

** 15'x¼" column (0.75/0.25 w/w% packed with DEGS/EGGS-X on chromosorb -W
N_2 flow 60 ml/min.

* 6'x 1/8" column (0.7/0.3 w/w% packed with DEGS/EGGS-X on chromosorb -W
N_2 flow 25ml/min

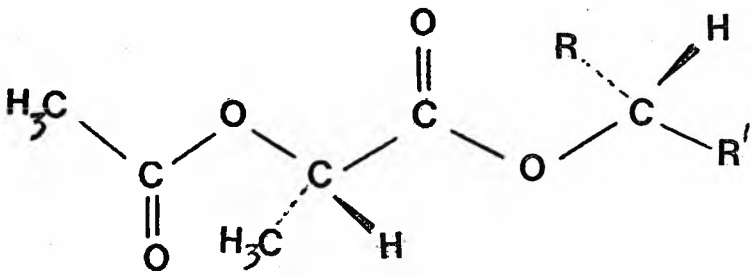
* present work; ** Halpern et al's (54) work.

X $\Delta(\Delta G^{O})$ depends on the resolution factor and the separation temperature hence even though the resolution factors are same the $\Delta(\Delta G^{O})$ vary in the above cases, due to different separation temperatures.

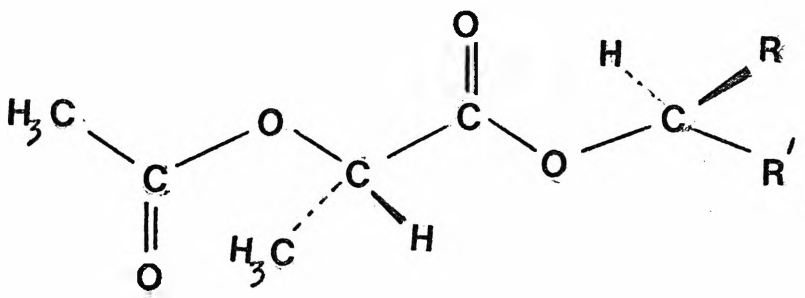
of the solute-solvent interactions between the liquid stationary phase and the diastereoisomer which is influenced by the steric environment of the functional groups of the diastereoisomer concerned. Hence different degrees of interactions with the stationary phase will occur where the functional groups of diastereoisomers are in different environments. The stationary phases DEGS and EGS-X are polyesters of ethylene glycol and succinic acid and a copolymer of ethylene glycol, succinic acid and silicone respectively. These polymers have no chirality and so their carbonyl groups would have equal interaction with the -NH group of each diastereoisomer if there is any H-bonding interactions. The different spatial arrangements of the atoms of the diastereoisomers are such that in each diastereoisomer only a few are related to one another as an object to the mirror image. Consequently the intermolecular forces between the diastereoisomers and the stationary liquid phase will be different resulting in different retention times. (Karger et al[56] have explained the resolvability of the column in terms of the configurational accessibility of the key functional group of the isomers which is available for interaction with the stationary phase. The authors[56] have reasoned that in compound A (SR-form)

the methyl group and one hydrogen atom lie on the same plane while the R group and the other hydrogen atom lie on the other side of the plane passing through the key ester functional group, and in compound B (SS-form) the two hydrogen atoms lie on the same plane whereas the bulkier methyl and R group lie on the opposite side of the plane passing through the ester group. Changing the chirality in this case switches the H and R groups (figure 9) thus enabling the ester group of the SS form to be in a less sterically crowded form and hence more accessible for interaction with the stationary phase. This configurational arrangement indicates that the key ester function in compound B (the SS-form) is more accessible for interaction with the stationary phase and hence has a higher retention time. Karger's observations are explained in figure (9).

Figure (9) Karger's observation of configurational accessibility.



B (SS-form)



A (SR-form)

Background of the Analytical Method

Our next objective was to establish a quantitative experimental procedure for following the enzyme hydrolysis. To this end we examined the possibility of using a G.L.C. method based on a qualitative study by Halpern et.al.[54] in which the progress of an enzyme hydrolysis is followed by extracting the substrates into an organic solvent, derivatizing and analyzing by G.L.C. The only disadvantage of this technique for following the enzyme hydrolysis is that it involves a discontinuous method as samples have to be withdrawn periodically from the reaction medium. However, the unique feature of the G. L. C. separation of the diastereoisomeric substrates makes it possible to examine the stereospecific action of the enzyme directly.

There are a number of areas where errors are likely to be introduced into such an experimental process. These include substrate solubility, the possibility of losses during the extraction procedure, substrate losses due to incomplete derivatization and the difficulty of carrying out a

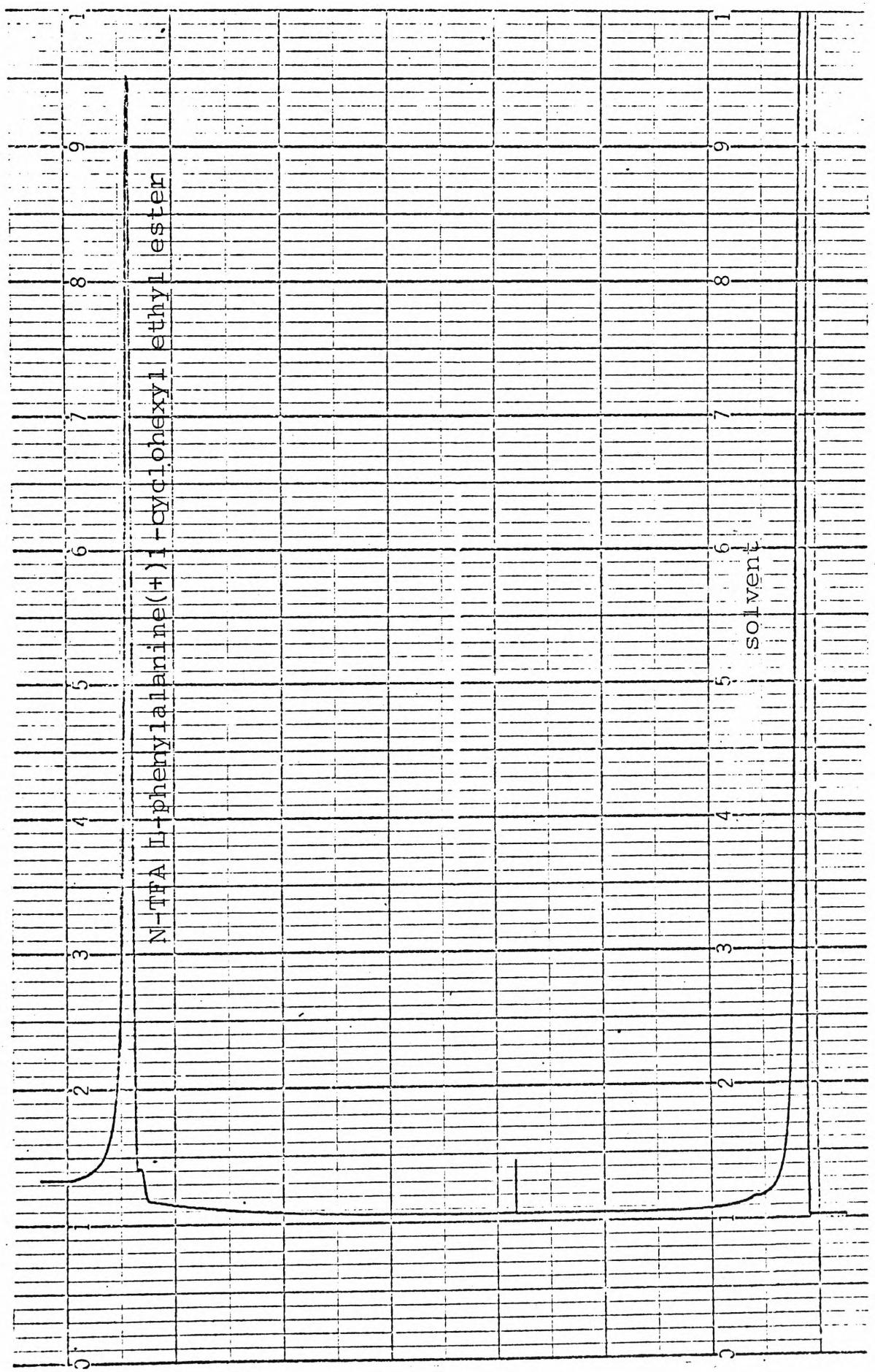
quantitative measurement of the detector response on a chart recorder. The losses during the extraction of the unhydrolyzed substrate will be mainly due to incomplete extraction and these may be compensated for by the use of an internal standard during the extraction process. The choice of a suitable internal standard requires that it should be stable in the reaction medium without being hydrolysed by the enzyme concerned and it must be completely soluble in the solvent employed in the extraction process. It is also desirable that the molecular weight of the internal standard be of the same order as that of the substrates in order to obtain a similar G. L. C. flame ionization detector response. It was shown that the menthyl ester of D-alanine, the benzyl ester of D-leucine and eicosane where suitable compounds for this purpose.

Substrate solubility in the buffer medium was another important factor which was examined carefully. To obtain good solubility the substrates

were shaken mechanically in the buffer medium and samples were analyzed periodically to determine the time required for complete dissolution. This varied from half an hour to more than two hours depending on the nature of the substrate. Longer periods are not desirable as non-enzymic hydrolysis may take place. The use of an ultrasonic shaking device at 40°C shortened the dissolution time considerably and homogeneous solutions were obtained in about 15 minutes. Higher molecular weight alkyl esters such as the menthyl and 2,2'-dimethyl-cyclohexyl esters could not be studied kinetically because homogeneous substrate solutions could not be obtained. Another major source of error could be introduced by incomplete derivatization for G. L. C. analysis. It had been reported[57] that the TFA derivatives are suitable derivatives for G. L. C. and that the derivatizations are quantitative under mild conditions. In order to check this L-phenylalanine-n-butyl ester was derivatised using trifluoroacetic

anhydride at room temperature and the reaction was monitored by T. L. C. The derviatives were shown to be quite stable at room temperature and these could be stored over molecular sieve 3A for some days. Accurate measurements of the detector response was possible using a suitable temperature programme and appropriate chart recorder speed. In this way sharp symmetrical peaks were obtained which could be measured accurately, Figure (10).

Figure (10) G.L.C. of L-phenylalanine(+)1-cyclohexyl ethyl ester (N-TFA derivative)



Application of Michaelis-Menten and Lineweaver-Burke Approach to α-Chymotrypsin Catalyzed Hydrolysis of Phenylalanine-Alkyl Ester Substrates

Rate equations of the Michaelis-Menten type have been widely used for the determination of K_m and k_3 values for many enzymic systems. Evidence from the literature[58-59] also suggests that the classical Michaelis-Menten behaviour would normally be expected for the α-chymotrypsin catalyzed hydrolysis of phenylalanine esters. The verification of the validity of the Michaelis-Menten approach to the systems presently under study was made through a series of kinetic investigations on the α-chymotrypsin-catalyzed hydrolysis of L-phenylalanine-n-butyl ester. Experimental data were collected over a wide range of initial substrate concentrations. Table (4) summarizes the initial rates of the reactions (v_0) determined at various initial substrate concentrations $[S]_0$ at 15°C. A plot of $[S]_0$ against (v_0) at 15°C gives a curve, figure (11) which shows the characteristic behaviour of a Michaelis-Menten mechanism.

From figure (11) K_m and k_3 were calculated for L-phenylalanine-n-butyl ester at 15°C; the values are given in table (5).

Table (4). Initial rates at varying initial substrate concentration for α-Chymotrypsin catalyzed hydrolysis of L-phenylalanine-n-butyl ester

$[S]_0$ Mol L^{-1} x 10^{-3}	(v_0) Mol L^{-1} Sec^{-1} x10^{-6}	$\frac{1}{[S]_0}$ x10^3	$\frac{1}{(v_0)}$ x 10^6
0.80	0.340	1.25	2.941
1.00	0.445	1.00	2.247
1.90	0.790	0.526	1.266
2.31	0.860	0.433	1.163
3.28	0.930	0.305	1.075
3.58	1.060	0.279	0.943
4.64	1.210	0.216	0.826
5.30	1.270	0.192	0.787
6.30	1.450	0.129	0.689

Experimental conditions: $15^{\circ}C$, pH 7.5

initial enzyme concentration

2.00×10^{-7} Mol L^{-1}

Figure (11). Michaelis-menten plot for L-phenylalanine -n-butyl ester at 15°C; $[E]_0$ = 2.00 x 10^{-7} Mol L^{-1}

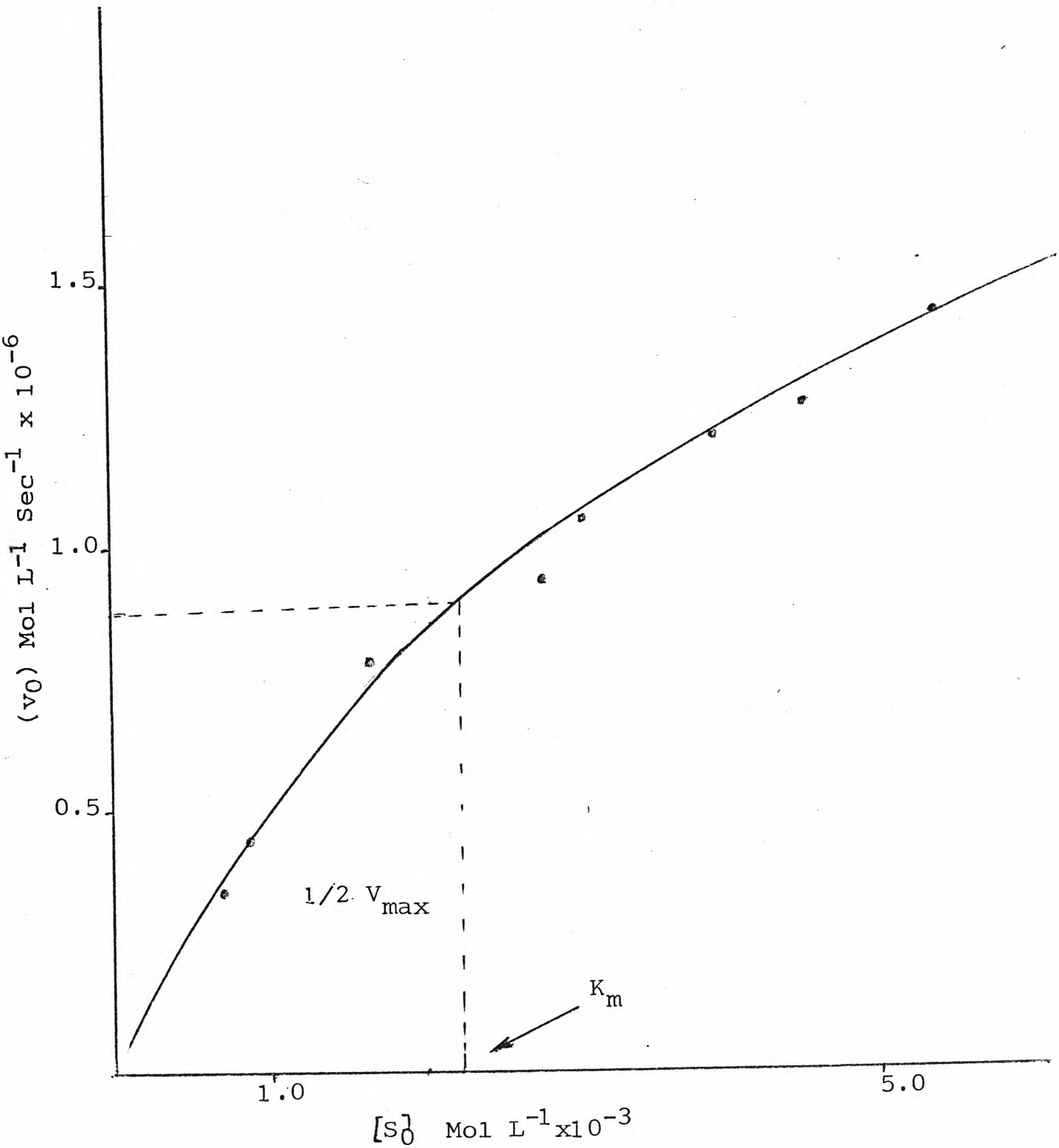


Table (5). Comparison of K_m and k_3 values for α-Chymotrypsin catalyzed hydrolysis of L-phenylalanine-n-butyl ester at 15°C. and pH 7.5

Method	$[E]_0 = 2.00 \times 10^{-7}$ Mol L^{-1}		$[E]_0 = 1.25 \times 10^{-7}$ Mol L^{-1}	
	K_m Mol $L^{-1} \times 10^{-3}$	k_3 Sec^{-1}	K_m Mol $L^{-1} \times 10^{-3}$	k_3 Sec^{-1}
Michaelis-Menten Plot	2.20 ± 0.40	8.00±.50	1.90±.20	9.60±.80
Lineweaver-Burke Plot	2.29± 0.26	8.92±.39	1.80±.12	10.60±.68

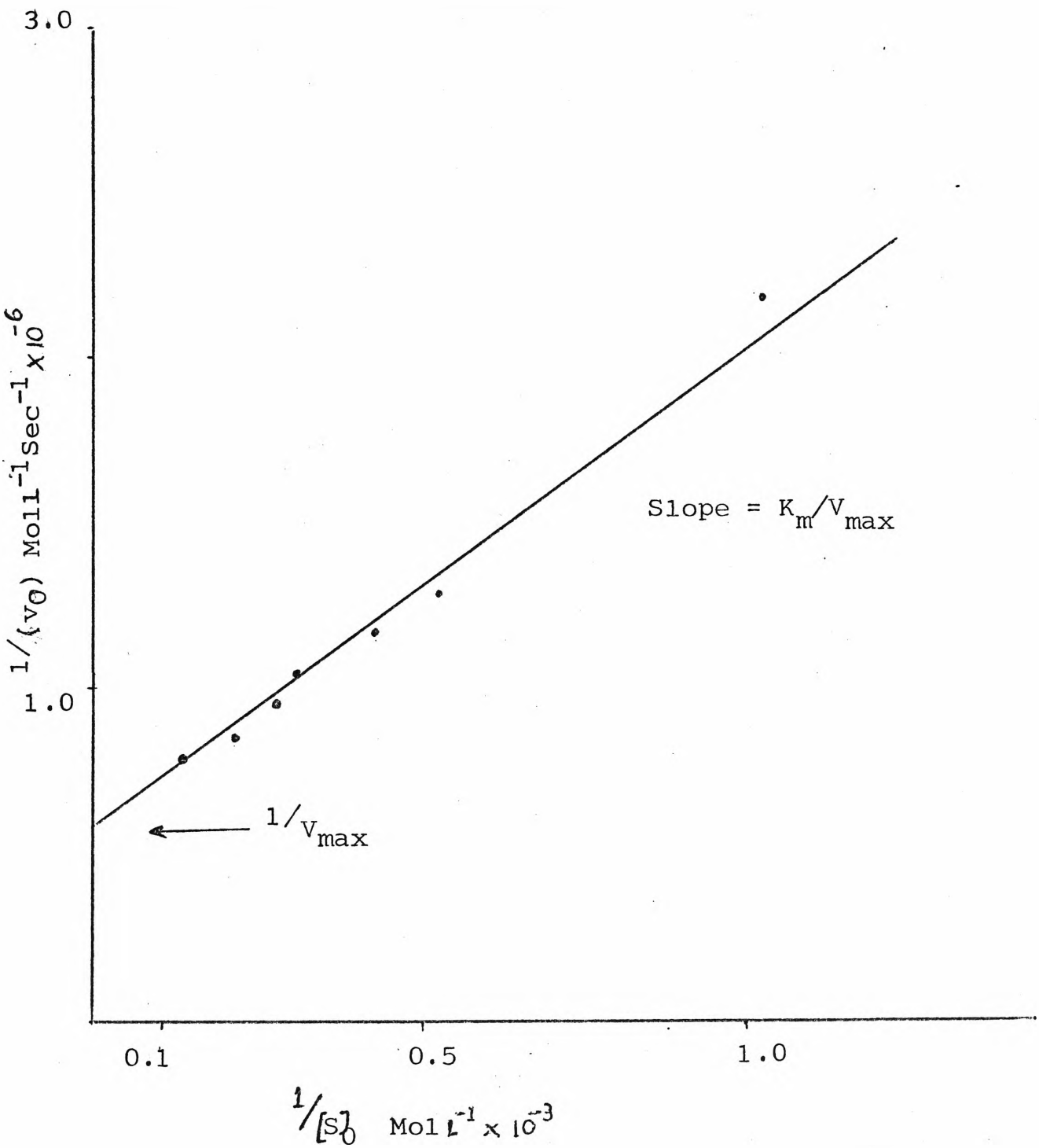
Determination of K_m and k_3 values from Michaelis-Menten plots are often not very accurate especially where low values of $[S]_0$ and (v_0) are involved. Lineweaver and Burke[11] demonstrated that the Michaelis-Menten equation in a linear form ;

$$\frac{1}{v_0} = \frac{K_m}{V_{max}} \left(\frac{1}{[S]_0} \right) + \frac{1}{V_{max}}$$

may often be used to give more accurate values of K_m and k_3. Figure (12) shows such a plot for the data of table (4). Table (5) gives a comparison of K_m and k_3 values obtained for L-phenylalanine-n-butyl ester at 15°C by the use of both Michaelis-Menten and the Lineweaver-Burke methods. It will be noted that there is agreement between the two methods within the estimated error of the results.

α-Chymotrypsin-catalyzed hydrolysis of L-phenylalanine-n-butyl ester at 15°C was also studied at a lower initial enzyme concentration than that used in the previously described study. It was observed that the plot of the initial substrate concentrations

Figure (12) Lineweaver-Burke plot for L-phenylalanine -n-butyl ester at 15°C; $[E_0] = 2.0 \times 10^{-7}$ Mol L^{-1}



versus initial rates table (6) of the reactions follows a similar Michaelis-Menten pattern figure (13) as was obtained in the previous case using a higher initial enzyme concentration. The values of K_m and k_3 calculated for this system are given in Table (5) and the Lineweaver-Burke plot is shown in Figure (14).

Again good agreement was observed in the value of K_m calculated by the two methods. Furthermore the calculated values of K_m are seen to be independent of initial enzyme concentration within experimental error.

Table (6). Initial rates of reactions at varying initial substrate concentrations for α-chymotrypsin catalyzed hydrolysis of L-phenylalanine-n-butyl ester

$[S]_0$ Mol L^{-1} $x10^{-3}$	(v_0) Mol L^{-1} Sec^{-1} $x10^{-6}$	$[S]_0^{-1}$ $x10^{3}$	$(v_0)^{-1}$ $x10^{6}$
1.49	0.59	0.67	1.69
2.00	0.685	0.50	1.46
3.00	0.83	0.33	1.20
4.00	0.93	0.25	1.08
5.36	1.037	0.186	0.98
8.00	1.15	0.125	0.87

Experimental conditions : 15°C, pH 7.5

initial enzyme concentration

1.25×10^{-7} Mol L^{-1}

Figure (13) Michaelis-Menten plot for L-phenylalanine -n-butyl ester at 15°C; $[E]_0 = 1.25 \times 10^{-7}$ Mol L^{-1}

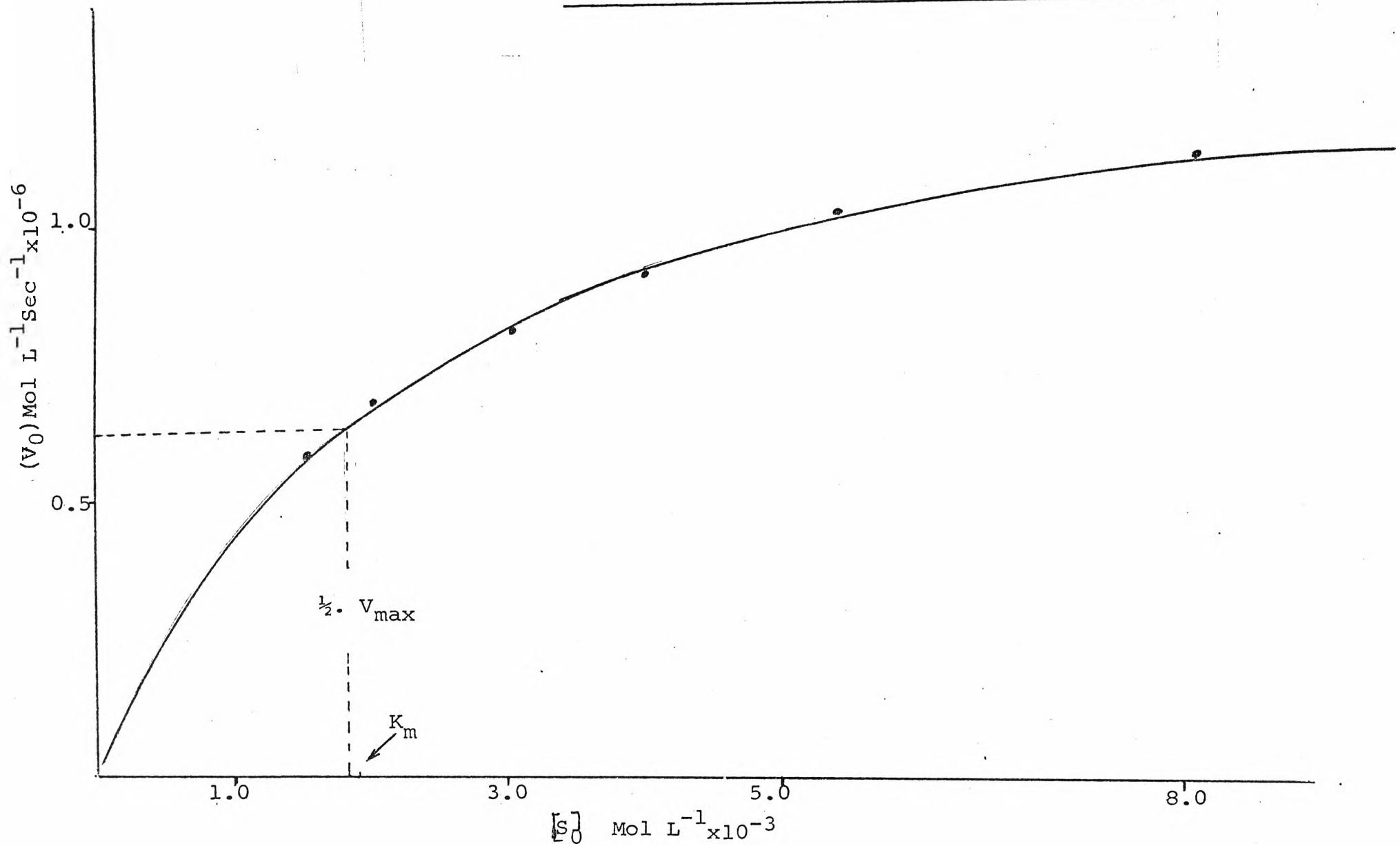
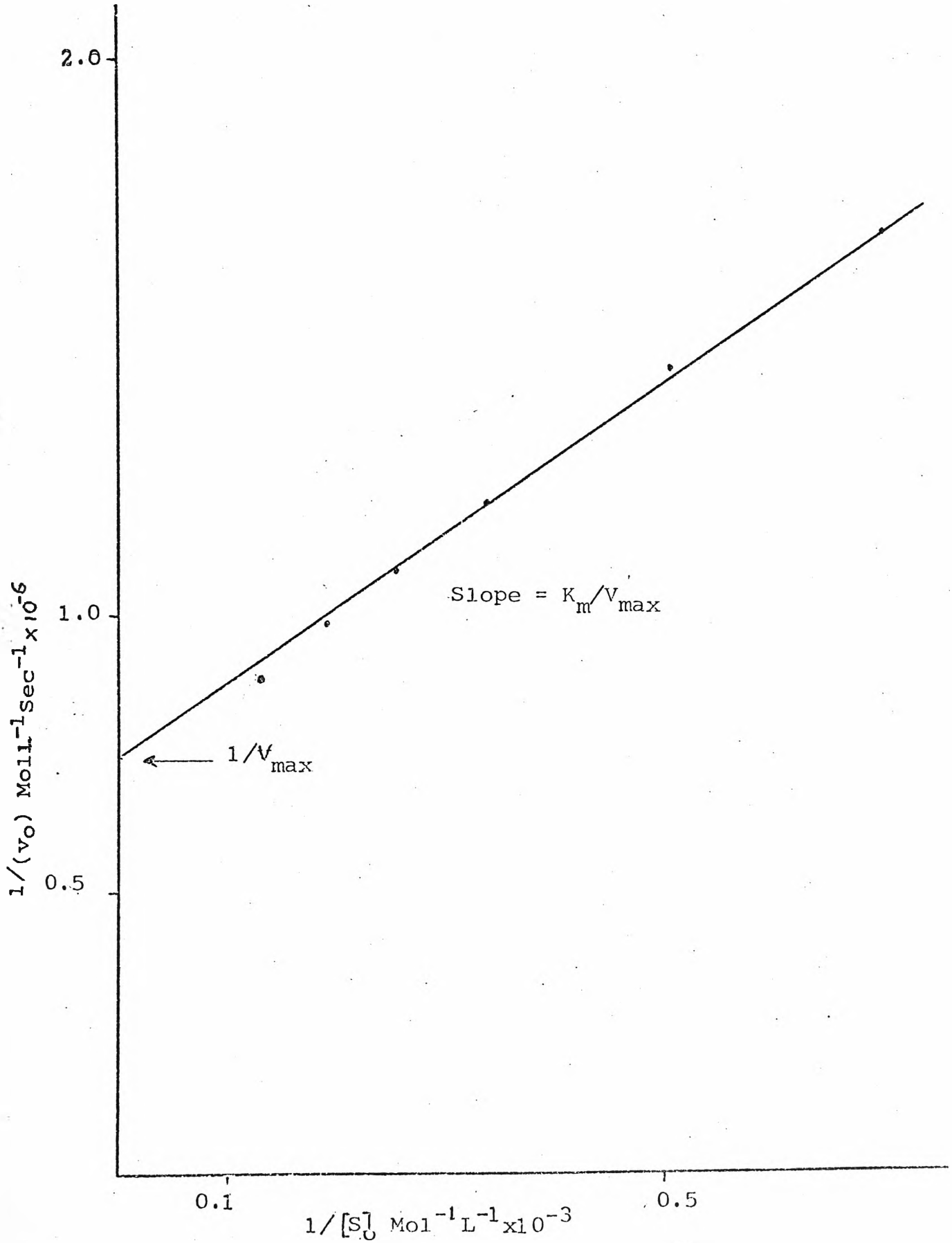


Figure (14). Lineweaver-Burke plot for L-phenylalanine -n-butyl ester at 15°C; $[E]_0 = 1.25 \times 10^{-7}$ Mol L^{-1}



A kinetic study of α-chymotrypsin-catalyzed hydrolysis of L-phenylalanine-n-butyl ester at 37°C revealed a similar Michaelis-Menten behaviour. Table (7) gives the values of the initial rates of the reaction obtained at varying initial substrate concentrations. The values of K_m and k_3 calculated by Michaelis-Menten plot figure (15) and by Lineweaver-Burke treatment figure (16) are summarized in Table (8). Once again the values calculated by the two methods are seen to agree.

Table (7). Initial rates of the reaction at varying initial concentration for α-chymotrypsin catalyzed hydrolysis of L-phenylalanine-n-butyl ester

$[S]_0$ Mol L^{-1} x10^{-3}	(v_0) Mol L^{-1} Sec^{-1} x10^{-6}	$\frac{1}{[S]_0}$ x10^3	$\frac{1}{(v_0)}$ x 10^6
2.00	1.16	0.500	0.86
2.960	1.41	0.338	0.71
3.500	1.65	0.286	0.606
4.80	1.90	0.208	0.53
5.00	1.95	0.200	0.513
6.60	2.10	0.152	0.476
10.00	2.90	-	-
15.00	2.20*	-	-

Experimental conditions : 37°C, pH 7.5

Initial enzyme concentration

2.00×10^{-7} Mol L^{-1}

* A decline in the rate of the reation was observed probably due to substrate inhibition at that high $[S]_0$ (Ref. (60)).

Figure (15). Michaelis-Menten plot for L-phenylalanine-n-butyl ester at 37°C; $[E]_0$ = 2.00x10^{-7} Mol L^{-1}

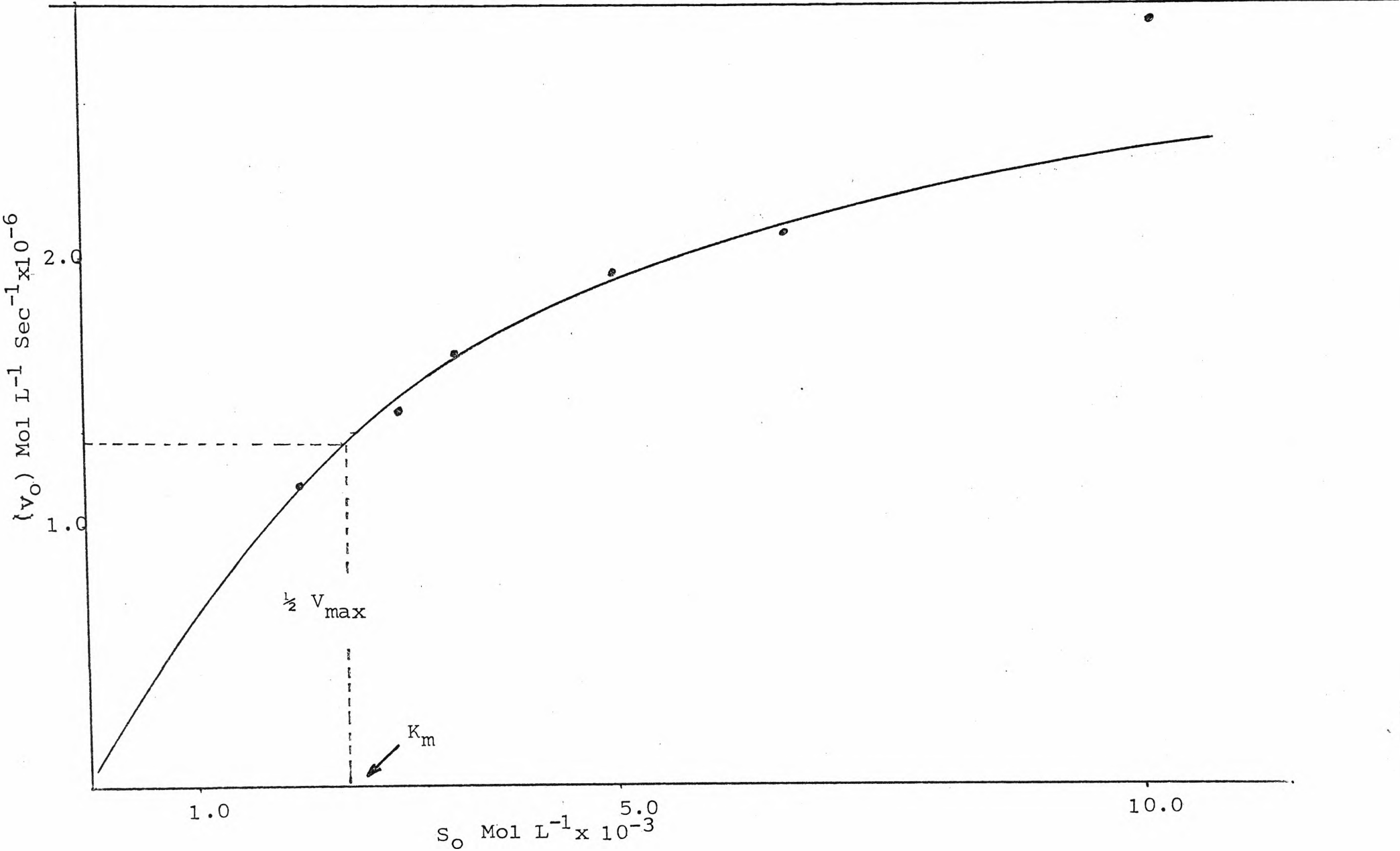


Figure (16). Lineweaver-Burke plot for L-phenylalanine -n-butyl ester at 37°C; $[E]_0 = 2.00 \times 10^{-7}$ Mol L^{-1}

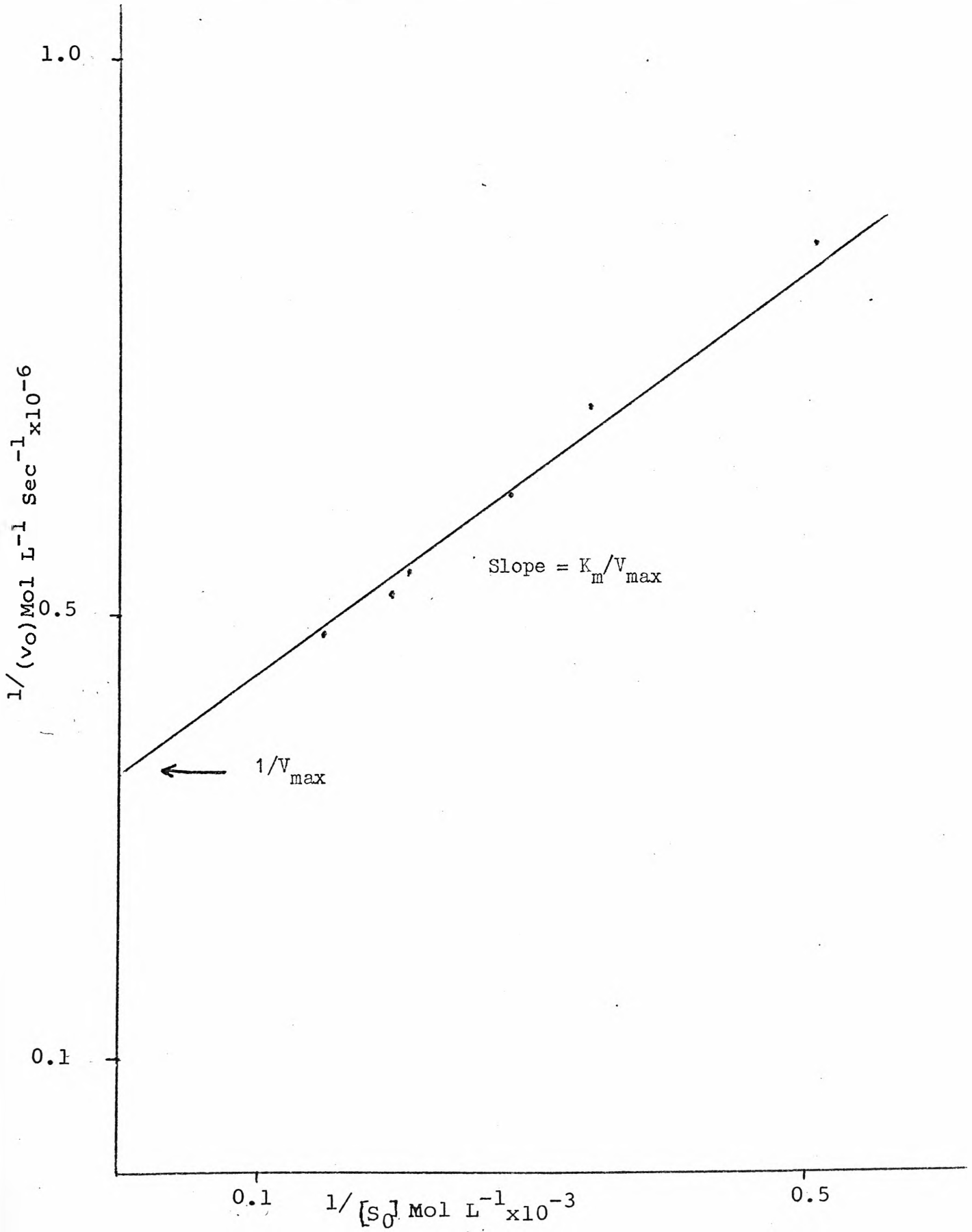


Table (8). Comparison of K_m and k_3 values for α-chymotrypsin catalyzed hydrolysis of L-phenylalanine -n-butyl ester at 37°C. and pH 7.5

Method	$[E]_0 = 2.00 \times 10^{-7}$ Mol L^{-1}		$[E]_0 = 1.2 \times 10^{-7}$ Mol L^{-1}	
	K_m Mol $L^{-1} \times 10^{-3}$	k_3 Sec^{-1}	* K_m Mol $L^{-1} \times 10^{-3}$	* k_3 Sec^{-1}
Michaelis-Menten plot	2.25±.25	11.50±.25	2.18±.38	10.50±.65
Lineweaver-Burke plot	2.49±.27	13.15±.30	2.25±.25	11.45±.55

* Results evaluated on the basis of three sets of experimental runs only.

Alternative Methods for the Evaluation of k_3 & K_m

The classical method for the determination of K_m and k_3 is rather time consuming as kinetic experiments must be conducted over a wide range of initial substrate concentrations. It was therefore one of our objectives to explore the possibility of developing an alternative analytical method for evaluating K_m and k_3. Two possibilities were examined.

1. Use of the Linear Integrated Michaelis-Menten Equation

The Michaelis-Menten equation can be written in an integrated form as:

$$k_3 [E]_0 = \frac{1}{t} \left\{ K_m \ln \frac{[S]_0}{[S]} + ([S]_0 - [S]) \right\}$$

Recasting the equation into a linear form we have,

$$\frac{1}{t} \ln \frac{[S]_0}{[S]} = \frac{k_3 [E]_0}{K_m} - \frac{1}{K_m} \left(\frac{[S]_0 - [S]}{t} \right)$$

Hence a plot of $\frac{1}{t} \ln \frac{[S]_0}{[S]}$ against $\left(\frac{[S]_0 - [S]}{t} \right)$ would result in a straight line with slope = $- 1/K_m$.

Thus this method would permit the evaluation of both K_m and k_3 from the data of a single experimental measurement. Table (9) summarizes the values of K_m and k_3 obtained for L-phenylalanine-n-butyl ester at varying experimental conditions.

Table (9). Values of K_m and k_3 obtained for L-phenylalanine-n-butyl ester by the use of Linear form of integrated Michaelis-Menten equation at varying experimental conditions. (at pH 7.5)

$[S]_0$ Mol L^{-1} x10^{-3}	$[E]_0$ Mol L^{-1}	temp ^0C	K_m Mol L^{-1} x 10^{-3}	k_3 Sec^{-1} **
1.49	$1.25x10^{-7}$	15^o	2.62± .41	4.00±
2.00	"	"	2.39± .18	2.72±
4.00	"	"	2.84± .52	2.56±
5.36	"	"	6.25±1.76	8.08±
8.00	"	"	8.22± .92	1.04±
1.90	$2.0x10^{-7}$	15^0	8.6 ± .94	18.21±
2.31	"	"	*	*
3.58	"	"	*	*
4.64	"	"	4.86±1.40	*
5.30	"	"	6.66± .83	2.85±
2.00	$2.0x10^{-7}$	37^o	*	*
2.96	"	"	*	*
3.50	"	"	*	15.5±
6.60	"	"	*	*
5.00	"	"	*	*
6.61	$1.25x10^{-7}$	37^o	*	*
2.96	"	"	*	*

* The calculated errors were greater than the values obtained hence are not recorded.

** Owing to a large standard error estimation the values were ignored.

2. Use of an extension of integrated Michaelis-Menten equation

The results in Table (9) show an inconsistency in the values of K_m and k_3 obtained by the use of the linear form of integrated Michaelis-Menten equation which utilizes the data obtained from a single experimental determination.

In an attempt to establish a more consistent method we have derived an equation which is virtually an extension of the integrated Michaelis-Menten equation (12). The integrated Michaelis-Menten equation can be expanded to analyze the data obtained from two individual experimental determination; the equations may be represented as

$$k_3[E]_0 = \frac{1}{t}\left[K_m \ln \frac{[S]_0'}{[S]'} + ([S]_0' - [S]')\right] \quad \text{.....Eq(17)}$$

and

$$k_3[E_0] = \frac{1}{t}\left[K_m \ln \frac{[S]_0''}{[S]''} + ([S]_0'' - [S]'')\right] \quad \text{...Eq(18)}$$

provided the experimental conditions in both the experimental determinations remain unchanged. Furthermore, the two equations (17)and (18)can be equated if the times of sampling for both the experiments

are the same. Hence,

$$K_m \ln \frac{[S]_0'}{[S]'} + ([S]_0' - [S]') = K_m \ln \frac{[S]_0''}{[S]''} + ([S]_0'' - [S]''$$

$$\text{or, } ([S]' - [S]'') = K_m \left(\ln \frac{[S]_0'}{[S]'} - \ln \frac{[S]_0''}{[S]''}\right) + ([S]_0' - [S]_0'' \quad \ldots \text{Eq (19)}$$

The above equation (19) is linear and a plot of $([S]' - [S]'')$ against $\left(\ln \frac{[S]_0'}{[S]'} - \ln \frac{[S]_0''}{[S]''}\right)$ would result in a straight line with slope = K_m. The value of K_m thus obtained can be substituted in either of the equations (17) or (18) and hence the value of k_3 can be determined. Table (10) gives the values of k_3 obtained in this manner for L-phenylalanine-n-butyl ester and Table (11) summarizes the values of K_m and k_3 obtained.

Table (10). Value of k_3 calculated for L-phenylalanine-n-butyl ester at $[S]_0'$ and $[S]_0''$ using the extended integrated Michaelis-Menten equation

$[E]_0$ Mol L^{-1}	temp ^0C	k_3 values			
		$[S]_0'$ Mol $L^{-1} \times 10^{-3}$	$k_3 Sec^{-1}$	$[S]_0''$ Mol $L^{-1} \times 10^{-3}$	$k_3 Sec^{-1}$
1.25×10^{-7}	37^0	6.60	10.8±.80	2.96	10.8±.90
1.25×10^{-7}	15^0	4.00	8.3±.40	1.49	8.10±.50
		8.00	9.95±.40	2.00	9.45±.40
		5.36	7.98±.25	1.49	7.52±.55
2.0×10^{-7}	37^0	5.00	14.2±1.00	2.00	14.2±1.00
		6.60	10.1±.60	2.96	9.9±.90
		6.60	11.4±.50	3.50	11.4±.50
2.0×10^{-7}	15^0	5.50	7.2±.50	2.00	7.14±1.20
		4.64	7.7±.60	1.90	7.6±.80
		5.30	6.68±.82	1.90	7.24±.98

Table (11). Determination of K_m and k_3 at various pairs of concentrations for L-phenylalanine-n-butyl ester by the use of extended integrated Michaelis-Menten equation.

$[S]_0$ pairs (Mol L^{-1})		$[E]_0$ Mol L^{-1}	temp °C	K_m Mol L^{-1} x10^{-3}	k_3 Sec^{-1}*
$[S]_0'$ x10^{-3}	$[S]_0''$ x10^{-3}				
6.60	2.96	1.25x10^{-7}	37°	2.62±.24	10.8±.85
5.00	2.00	2.0x10^{-7}	37°	2.10±.16	14.2±1.0
6.60	2.96	"	"	2.10±.23	10.0±.75
6.60	3.50	"	"	2.05±.21	11.4±.50
4.00	1.49	1.25x10^{-7}	15°	2.10±.29	8.2±.45
8.00	2.00	"	"	1.33±.15	9.7±.40
5.36	1.49	"	"	1.80±.03	7.75±.40
5.50	2.00	2.0x10^{-7}	15°	2.39±.18	7.17±.85
4.64	1.90	"	"	1.91±.28	7.65±.70
5.30	1.90	"	"	2.27±.20	6.96±.90

* Mean values from Table (10).

Observation of the values obtained for both K_m and k_3 utilizing the two analytical methods, linear integrated Michaelis-Menten equation and the extension of integrated Michaelis-Menten equation described above suggests that the treatment of experimental data by the use of the extension of integrated Michaelis-Menten equation gives values for K_m and k_3 which are more in agreement with the data obtained by the classical Michaelis-Menten approach and the Lineweaver-Burke method within experimental errors. Table (12) gives a collective comparison of the values obtained for K_m and k_3 by Michaelis-Menten, Lineweaver-Burke and by the use of an extension of integrated Michaelis-Menten equation for L-phenylalanine-n-butyl ester at various experimental conditions.

The values in Table (9) suggest that method 1 (Use of the linear form of Integrated Michaelis-Menten equation) is totally unsuitable for the evaluation of K_m and k_3 for this type of analytical system; though there has been a brief description of this type of method[60] as a possible source of calculating k_3 and K_m values but because, of its inaccuracy its further use has not been much explored.

Table (12). A Comparative analysis of K_m and k_3 values for L-phenylalanine-n-butyl ester obtained by the use of Classical methods and the extended integrated Michaelis-Menten equations

$[E]_0$ $Mol L^{-1} x10^{-7}$	Temp ^oC	Method					
		Michaelis-Menten		Lineweaver-Burke		*Extended Integrated Michaelis-Menten	
		$K_m Mol L^{-1} x10^{-3}$	$k_3 Sec^{-1}$	$K_m Mol L^{-1} x10^{-3}$	$k_3 Sec^{-1}$	$K_m Mol L^{-1} x10^{-3}$	$k_3 Sec^{-1}$
1.25	15	1.90±.20	9.60 ±.80	1.80±.12	10.60±.68	1.75±.16	8.55±.41
2.00	15	2.20±0.40	8.00±.50	2.29±.26	8.92±.39	2.19±.22	7.26±.66
1.25	37	2.18±.38	10.50±.65	2.25±.25	11.45±.55	2.62±.24	10.80±.85
2.00	37	2.25±.25	11.50±.25	2.49±.27	13.15±.30	2.10±.19	11.86±.75

* The values listed here are the average values calculated for different pairs of $[S]_0$ (from Table 11).

Leaving Group Specificity

It has now been well established[61] that the most significant part of α-chymotrypsin specificity lies with the carboxylic portion of the amino acid. In our attempt to study the effect of the leaving group on α-chymotrypsin catalyzed hydrolysis of phenylalanine alkyl esters we have synthesised a series of alkyl esters of L-phenylalanine and subjected them to kinetic investigations. K_m and k_3 values for such substrates have been determined by the extension of integrated Michaelis-Menten eq as previously described and are summarised in table (14).

Bender et. al.[61] have studied the hydrolysis of several series of substituted phenyl esters by α-chymotrypsin. They have studied both the acylation and the deacylation steps individually and concluded that there is at least qualitative evidence that the presence of electron withdrawing substituents facilitates the acylation step. This observation indicates that the substituents with electronic effects influence the enzyme-substrate reaction at least in the initial binding stages.

Table (13) gives the values of k_3 determined by the use of equation (17 & 18) at two different initial substrate concentrations. A noticeable agreement in the k_3 values are obtained.

Table (13) Comparison of k_3 values obtained at two different initial substrate concentrations of L-phenylalanine alkyl esters at 15°C *

R	k_3 values at $[S]_0'$ and $[S]_0''$	
	k_3' sec^{-1}	k_3'' sec^{-1}
methyl	12.01 ±1.08	11.96 ± 1.34
ethyl	4.97 ±0.16	4.94 ± 0.28
n-propyl	6.53 ±0.90	6.28 ± 1.43
n-butyl	7.20 ±0.50	7.14 ± 1.20
n-amyl	4.71 ±0.15	4.67 ± 0.30
n-octyl	3.75 ±0.03	3.70 ± 0.22
cyclohexyl	3.50 ±0.04	3.48 ± 0.26
(-)2-methyl-1-butyl	4.32 ±0.07	4.27 ± 0.32
(-)2-octyl	2.39 ±0.04	-
(+)2-octyl	2.13 ±0.025	2.13 ± 0.002
(+)3-methyl-2-butyl	1.14 ±0.04	1.13 ± 0.05
(+)4-methyl-2-pentyl	3.56 ±0.34	-
(+) 1-cyclohexyl-ethyl	3.25 ±0.12	-
(+) 3,3'-dimethyl-2-butyl	1.31 ±0.03	1.30 ± 0.08

* Experimental condition pH = 7.5

$[E]_0 = 2.00 \times 10^{-7}$ Mol L^{-1}

Table (14) The K_m and k_3 values for various L-phenylalanine alkyl esters at 15°C *

R	K_m Mol L^{-1} $\times 10^{-3}$	k_3 sec^{-1} **
methyl	13.7 ± 1.8	11.98 ± 1.21
ethyl	6.4 ± 0.50	4.95 ± 0.22
n-propyl	4.24± 1.2	6.40 ± 1.16
n-butyl	2.19± 0.22***	7.17 ± 0.85
n-amyl	4.58± 0.43	4.69 ± 0.22
n-octyl	0.54± 0.09	3.73 ± 0.12
cyclohexyl	1.35± 0.21	3.49 ± 0.15
(-)2-methyl-1-butyl	1.62± 0.22	4.29 ± 0.20
(-)2-octyl	0.85± 0.06	2.39 ± 0.04
(+)2-octyl	0.71± 0.04	2.13 ± 0.025
(+)3-methyl-2-butyl	0.59± 0.11	1.13 ± 0.045
(+)4-methyl-2-pentyl	2.94± 1.20	3.56 ± 0.34
(+)1-cyclohexyl-ethyl	3.40± 0.72	3.25 ± 0.12
(+)3,3-dimethyl-2-butyl	1.24± 0.19	1.31 ± 0.05

* Experimental Conditions pH = 7.5

$[E]_0$ = 2.00×10^{-7} Mol L^{-1}

** k_3 values are mean values from Table (13).

*** K_m " " " " " " (11) at specified conditions.

We have explored the possibility of determining a relationship between the structure of the leaving group (in terms of M.W. of group R; Table 15) and K_m and k_3. Our initial observation revealed that a plot of k_3/K_m (which is a measure of enzyme specificity) against the molecular weight of the leaving group of the simple straight chain homologous series of L-phenylalanine alkyl esters show a linear trend, hence subsequent effort was made to correlate the log terms of K_m and k_3/K_m. It was found that a plot of log K_m against the M.W. gave a straight line in all cases of studied substrates with the exception of the amyl ester. The reason of this exception is unknown and could be characteristic of the amyl ester. The significant aspect of this analysis is that since K_m is a measure of the equilibrium between the substrate and the enzyme, log K_m will therefore give a measure of the free energy change for the formation of the enzyme substrate complex and consequently would indicate the degree of bonding between the enzyme and the substrate. Table (15) refers to the molecular weight of the leaving groups in a homologous series of alkyl esters of L-phenylalalnine and the corresponding log K_m values calculated by the use of the extended integrated Michaelis-Menten equation.

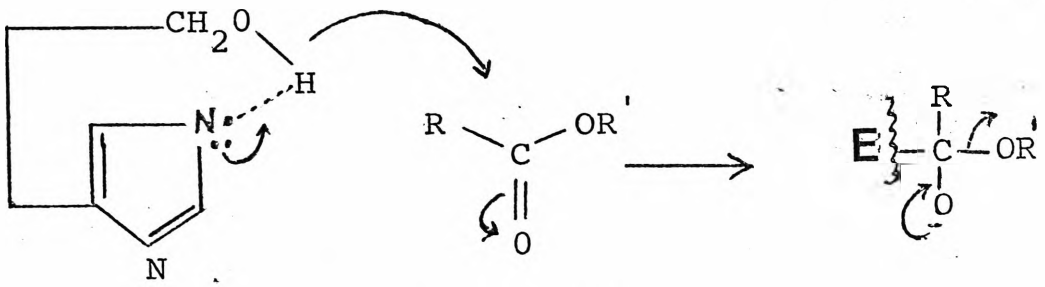
Table (15) The M.W. of Leaving group and LOG k_3/K_m values for simple straight chain homologous L-phenylalanine alkyl esters.

R	M.W	k_3/K_m	LOG k_3/K_m	LOG K_m
methyl	15	874.5	2.94	- 1.86
ethyl	29	773.4	2.89	- 2.19
n-propyl	43	1509.4	3.18	- 2.37
n-butyl	57	3259.0	3.51	- 2.66
n-amyl	71	1024.0	3.01	- 2.34
n-octyl	113	6907.4	3.84	- 3.27

Esters with branched chain leaving groups were found to deviate from the above observation. A number of reasons could be attributed for such observation, namely, the steric factors arising from the substitution at the α-Carbon atom adjacent to the reactive centre of the substrate (figure 17), and the thermodynamic factor namely the entropy which is associated with a structural or environmental change during the course of the reaction. Our discussion is mainly concerned with the structural features of the substrate or the substituent effect arising from the crowding at the substrate reactive site and no attempt has been made to evaluate the entropy changes as it may be assumed that the overall influence of such factor may not vary to a large extent in systems where large changes in the rotational entropy due to the involvement of a ring closure may be counterbalanced by a decrease in the overall entropy due to the increase in the molecular weight of the leaving group. It will be observed from the data in Table (16) that the specificity constants (k_3/K_m) of the L-phenylalanine-2-octyl ester substrates decreases by a factor of two as compared to the straight chain n-octyl ester. Similar trend is observed in the butyl series of esters where the 3-methyl-2 butyl and the 3,3'-dimethyl-2 butyl esters show a decreasing order

of the specificity constants as compared to the straight chain L-phenylalanine-n-butyl ester substrate. These results tend to point towards the fact that the initial reaction of α-chymotrypsin and substrates with branched chain leaving groups (predominantly branching at the α-carbon atom nearest to the reactive site) is affected by the branching at the α-carbon atom even though it may be agreed that free roation around the C-O bond could reduce to some extent the steric or non bonding interactions (figure 17).

Figure (17). The effect of structure on α-chymotrypsin reactivity.

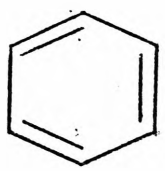


(reactive form of α-chymotrypsin)

(Substrate)

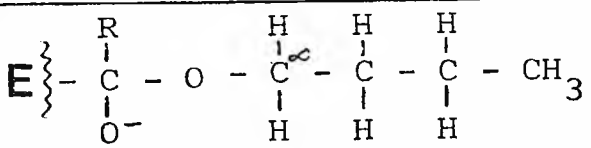
(cf. mechanism of α- chymotrypsim reaction p.28)

for phenylalanine alkyl ester substrates

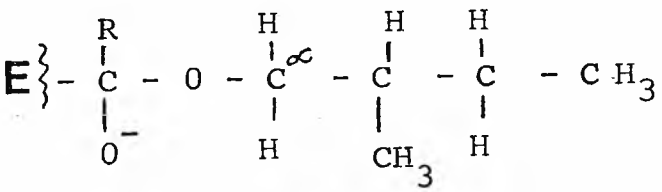
R =  $-CH_2-CH(NH_2)-$ (R' = alkyl leaving group)

Structure of the butyl series of esters studied

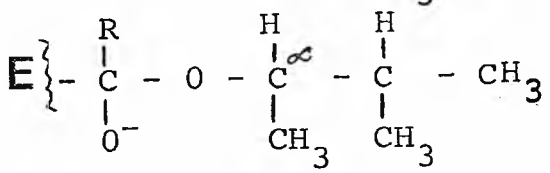
Compound 1
n-butyl group



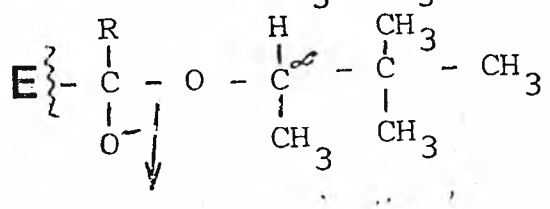
Compound 2
(-)2-me-1-butyl group



Compound 3
(+)3-methyl-2-butyl



Compound 4
(+)3,3'-dimethyl-2-butyl

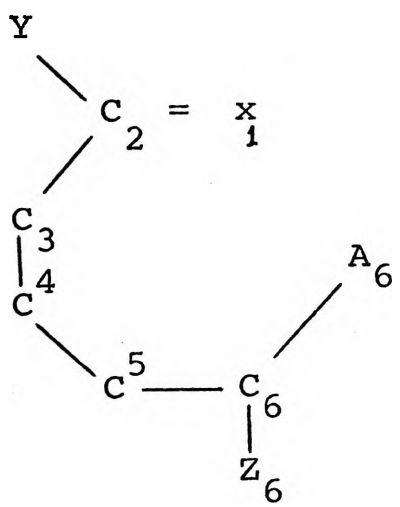


(C - O bond referred in the discussion)

(62)

The Application of rule of Six

The rule of six is an empirical rule which has been applied particularly to explain the rates of esterification and to some extent hydrolysis of esters and amides. The rule states that the steric effect offered by a compound during an addition reaction involving a double bond will increase with the increasing number of atoms in the six position. The numbering is calculated starting from the reaction site.



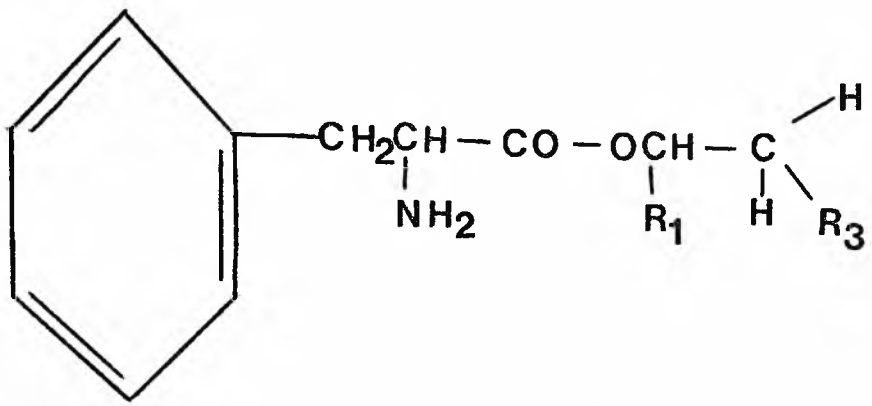
We have applied this rule to L-phenylalanine alkyl ester substrates in order to investigate any correlation between the structure and reactivity from the point of view of the number of atoms at the six position. The table (16) gives a listing of substrates with the kinetic parameters. The lack of correlation with the number of atoms at the six position suggests that the steric interactions between α-chymotrypsin and L-phenylalanine alkyl esters investigated here appear to be minimal.

Table (16). Six numbers of some phenylalanine alkyl esters

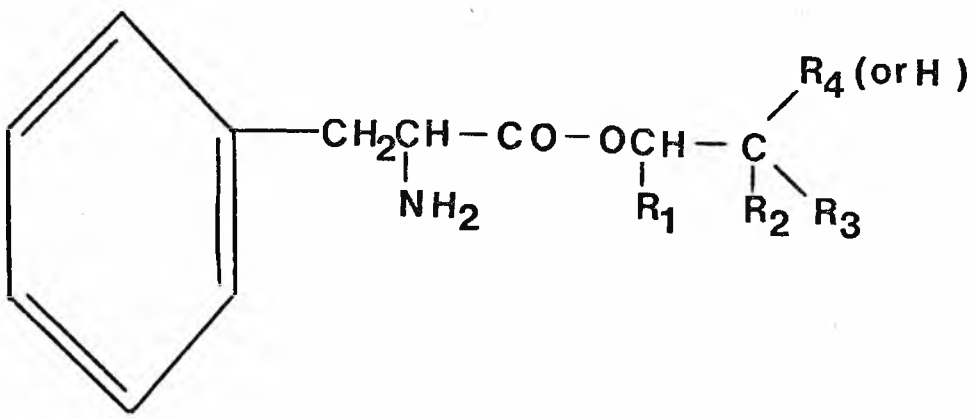
R	Six number C	H	Total	K_m Mol L^{-1} x10-3	k_3 sec^{-1}	k_3/K_m
methyl	0	0	0	13.7±1.80	11.98±1.21	874.50
ethyl	0	0	0	6.4± .50	4.95± .22	773.40
n-propyl	0	3	3	4.24±1.20	6.40±1.16	1509.40
n-butyl	1	2	3	2.19± .22	7.17± .85	3259.00
n-amyl	1	2	3	4.58± .43	4.69± .22	1024.00
(-) 2-methyl-1-butyl	1	2	3	1.62± .22	4.29± .20	2648.15
(+) 3-methyl-2-butyl	0	6	6	0.59± .11	1.13±.045	1915.25
(+) 3,3'dimethyl-2-butyl	0	9	9	1.24± .19	1.31± .05	1056.45
(+) 4-methyl-2-pentyl	2	1	3	2.94±1.20	3.56± .34	1210.90
cyclohexyl	-	-	-	-	-	-
n-octyl	1	2	3	0.54± .09	3.73± .12	6907.40
(-) 2-octyl	1	2	3	0.85± .06	2.39± .04	2811.80
(+) 2-octyl	1	2	3	0.71± .04	2.13±.025	3000.00

Stereospecificity studies on diastereoisomeric L-phenylalanine (±)alkyl esters

The primary sterospecificity of α-chymotrypsin has been studied extensively and the enzyme has been shown specifically to be L-directing[40]. The secondary specificity of the enzyme with respect to the diastereoisomeric alcohol moieties of the ester substrates however,has not been fully explored. Hence,we have examined the stereospecific action of α-chymotrypsin on diastereoisomeric L-phenylalanine (±)alkyl esters. This was done by exposing the synthetic substrates to α-chymotrypsin catalyzed hydrolysis. K_m and k_3 values were calculated for the individual diastereoisomers in the diastereoisomeric mixture by the method described before,namely the extended integrated Michaelis-Menten equation (Page 80)and are summarized in Table(18). The values suggest that the structure and the configuration of the substrate plays an important role in determinining which of the two isomers in the diastereoisomeric mixture would be a better substrate for the enzyme. For example the L-phenylalanine(+)alkyl ester of b,c,d,and e (Table 18) are more readily hydrolyzed than the corresponding L-phenylalanine (-)alkyl esters, whilst the L-phenylalanine (-)alkyl esters of a and f (Table 18) are more readily attacked. Hence a generalized conclusion from the above observation may be drawn, that, the L-phenylalanine (-)alkyl esters of general structure I are more reactive than the L-phenylalanine(+)alkyl esters; but the reverse is true with substrates of general structure II.

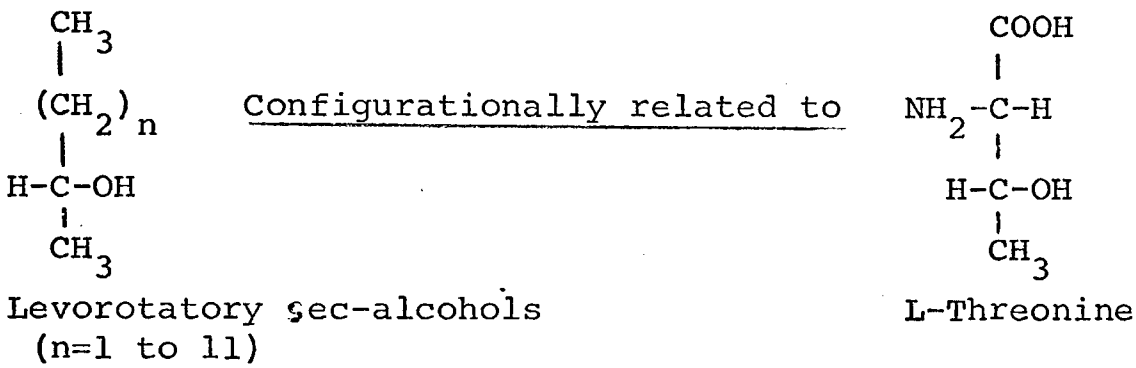


(Structure I)



(Structure II)

Extending this observation to the configuration of the alcohol moieties of the esters and the experimentally observed reactivity of the diastereoisomers it may be concluded that since a number of (-)alcohols (structure type I table 17)have already been related to the L-configuration of α-aminoacids[63], the L-phenylalanine(-) alkyl esters (or the L-L-diastereoisomer) is the preferred substrate.



Levorotatory sec-alcohols (n=1 to 11)

L-Threonine

In the present investigation of the G.L.C.analysis of the diastereoisomeric L-phenylalanine(±)alkyl esters one of the most interesting results was the observed regularity in the order of elution of the diastereoisomers. Esters derived from the (+) alcohols had a larger retention time as compared to the esters derived from the corresponding (-)alcohols. Gil Av and Nurok[55] have suggested an emperical relationship between the order of elution and configuration at the asymmetric centre. Thus for N-TFA-α-aminoacid-2-alkyl esters the LD-diastereoisomers have larger retention times than the LL-diastereoisomers.This order of elution was varified using different columns[55].Further proof was added by

observing the order of elution of L-phenylalanine(+) and (-)2-octyl and 2-butyl esters derived from configurationally know (+) and (-)2-octanols and 2-butanols[64,65].

Table (17) Diastereoisomeric L-phenylalanine(±)alkyl ester structure and reactivity relationship

(±)alkyl groups	Structure type	R_1	R_2	R_3	R_4	diastereoisomeric configuration	diastereoisomeric reactivity
2-butyl	I	CH_3	H	CH_3	H	LL	(-)isomer
2-methyl-cyclohexyl	II	*	*	CH_3	H	-	(+)isomer
3-methyl-2-butyl	II	CH_3	CH_3	CH_3	H	LD	(+)isomer
3,3-dimethyl-2-butyl	II	CH_3	CH_3	CH_3	CH_3	LD	(+)isomer
3-methyl-2-pentyl	II	CH_3	CH_3	C_2H_5	H	LD	(+)isomer
4-methyl-2-pentyl	I	CH_3	H	C_3H_7	H	LL	(-)isomer
2-methyl-3-pentyl	II	C_2H_5	CH_3	CH_3	H	LD	(+)isomer

*R_1 and R_2 are the part of the same ring system.

This remarkable reversal of specificity observed for substrates of structure type II may be due to a conformational change in the enzyme active site during the initial binding stage to accommodate the more sterically hindered esters of type II.Table (17)summarizes the relation between the diastereoisomeric structure and the observed reactivity of α-chymotrypsin catalyzed hydrolysis of L-phenylalanine ($\pm$)alkyl esters.

Newman projection formulae[66] are highly useful representations of different arrangements of atoms or groups with in the molecule that can be converted into one another by rotation about single bonds. The molecules are viewed from front to back in the direction of the bond linking the asymmetric carbon atom.

Constituting Newman projection formulae for various enzyme-diastereoisomeric substrate complex a trend in the relationship among K_m,k_3 and the observed diastereoisomeric reactivity may be established. However,some exceptions are noticeable. For each diastereoisomer there are three conformations , six for the diastereoisomeric pair; they are represented in the figure (18). In constituting the Newman projections of the enzyme-substrate complexes no definite shape regarding the binding of the enzyme to the substrate is indicated and the enzyme surface is represented by a wavy line. This is because of the complexity of the

conformation of the enzyme active site. The oxygen atom of the O-alkyl group is bonded to the phenylalanine and is subjected to free rotation thus adding flexibility to the alkyl-enzyme interaction (figure 18). Furthermore, it is possible that there is some sort of bonding (possibly H-bonding) between the O-atom and the enzyme.

In most cases of the studied substrates it is found that diastereoisomeric pairs with alkyl- enzyme interaction exhibited by the Newman projections are associated with a large difference in both K_m and k_3 values. This is particularly observed in compounds of general structure II (noticeable exception compound g Table 20) while the Newman projections of compounds of general structure I indicated a zero alkyl-enzyme interaction. Table (20) summarizes the K_m, k_3 values for the diastereoisomeric L-phenylalanine (±) alkyl esters along with the most preferred Newman projection formula.

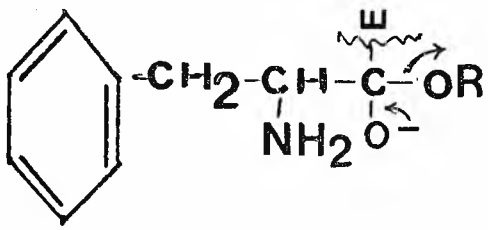
It may be suggested that the introduction of an equilibrium constant K representing the equilibrium

$$E + S \rightleftharpoons ES \longrightarrow E + P$$

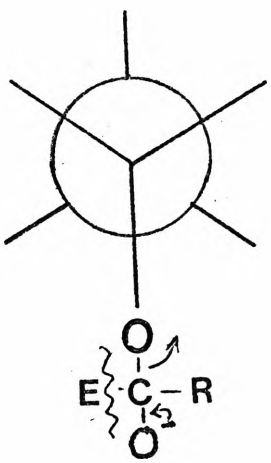
instead of K_m may decrease to a certain extent the above discrepancy, but this was not evaluated as K does not represent a true equilibrium constant since the formation of ES is followed by its decomposition.

Figure 18 Newman Projection formulae of L-phenylalanine alkyl esters

The general structural formula of the enzyme



← L-phenylalanine alkyl ester substrate complex is represented as (Newman projection)



(R ; cf figure 17)

The Newman projections of various L-phenylalanine alkyl esters are constituted as follows (only the alkyl part is shown)

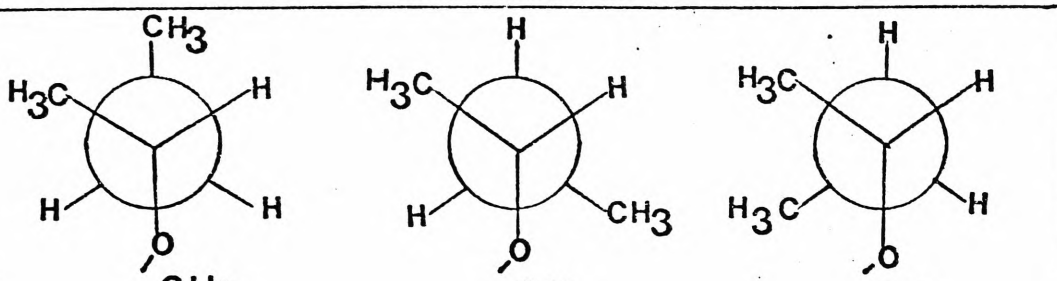
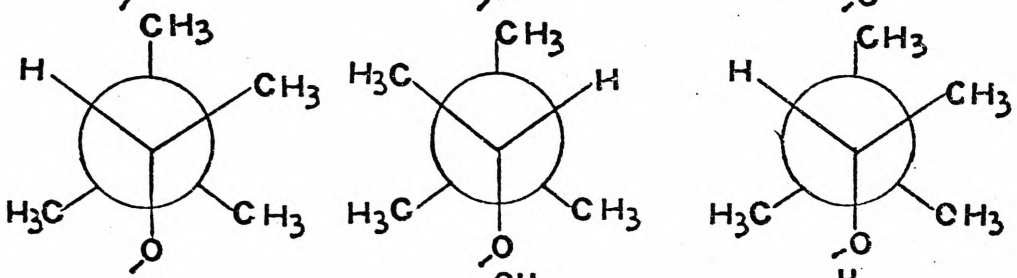
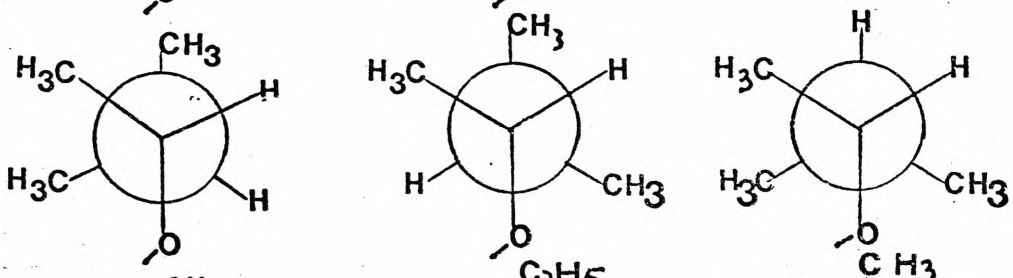
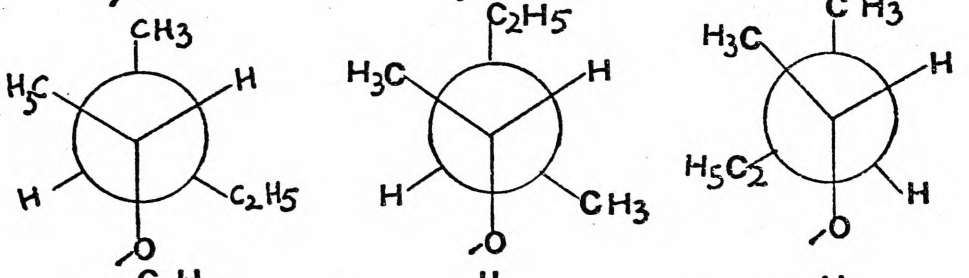
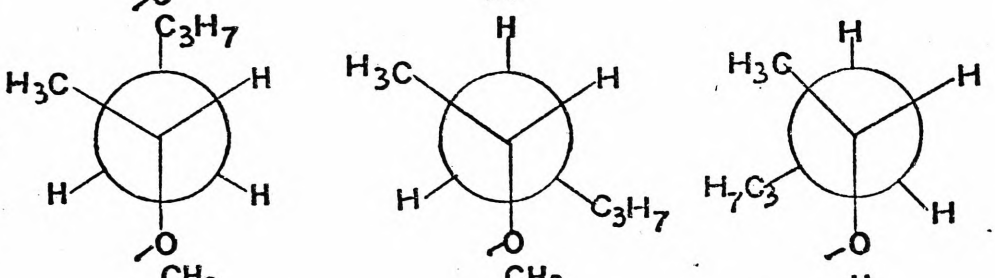
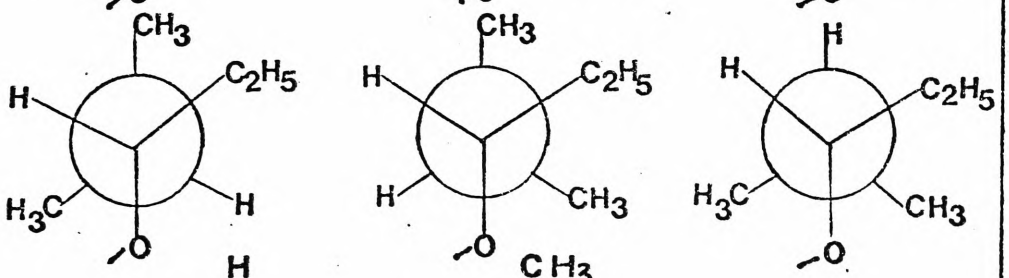
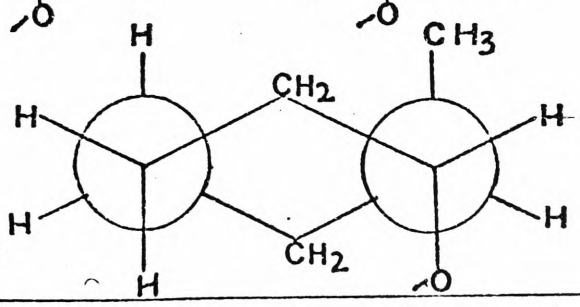
Alkyl Group	Conformations
2 - butyl	
3,3'-dimethyl -2-butyl	
3-methyl-2-butyl	
3-methyl-2-pentyl	
4-methyl-2-pentyl	
2-methyl-3-pentyl	
2-methyl cyclohexyl (trans isomer)	 ..... etc.

Table (19) summarizes the values of k_3 determined by the use of equation (17) or (18)p.80, at two different initial substrate concentrations; a good agreement in the values is obtained.

The large difference in the diastereoisomeric specificity constants and catalytic rate constants exhibited by a number of diastereoisomeric pairs (Table 18) suggested the feasibility of using α-chymotrypsin for the preparative resolution of diastereoisomeric L-phenylalanine(±)alkyl esters and hence lead to the synthesis of optically pure alcohols. Whilst yields were low, preliminary results suggest that this method may have some value for the small scale preparation of optically active alcohols.

Lin Palmer and Jones[67] have reported that the structure and chirality of the alcohol moieties affect both the catalytic and the binding constants of the substrates and that the greatest difference between enantiomeric alcohol moieties are observed for non-specific substrates for example of the dihydrocinnamate and hippurate series. The authors[67] have found that, by using the hippurate of racemic 2-butyl alcohol the stereospecificity of the alcohol site is such that sufficient resolution may be achieved.

Table (18). K_m and k_3 values calculated for L-phenylalanine (±)alkyl esters at 15°C and pH 7.5

	(±) R	K_m Mol $L^{-1}x10^{-3}$	k_3 sec^{-1} *	k_3/K_m
a	(+)2-butyl	0.73 ± 0.12	0.90 ± .025	1233 ± 145
	(-)2-butyl	0.67 ± 0.10	1.19 ± .09	1776 ± 114
b+	(+)2-methyl-cyclohexyl	1.16 ± 0.24	2.48 ± .25	2138 ± 188
	(-)2-methyl-cyclohexyl	2.28 ± 0.25	1.94 ±0.15	851 ± 25
c	(+)3,3′-dimethyl-2-butyl	1.40 ± 0.16	1.53 ±0.13	1093 ± 29
	(-)3,3′-dimethyl-2-butyl	2.10 ± 0.35	0.84 ±0.11	400 ± 12
d**	(+)3-methyl-2-butyl	2.37 ± 1.02	1.65 ±0.56	696 ± 44
	(-)3-methyl-2-butyl	1.09 ± .51	0.56 ±0.12	514 ± 89
e	(+)3-methyl-2-pentyl	1.55 ± 0.17	1.92 ±0.12	1239 ± 53
	(-)3-methyl-2-pentyl	1.12 ± .23	1.58 ±0.42	1411 ± 70
f	(+)4-methyl-2-pentyl	1.18 ± .26	1.15 ±0.13	975 ± 86
	(-)4-methyl-2-pentyl	1.32 ± .19	2.73 ±0.32	2068 ± 48
g	(+)2-methyl-3-pentyl	0.32 ± 0.17	0.68 ±0.06	2125 ± 615
	(-)2-methyl-3-pentyl	0.62 ± 0.18	0.64 ±0.04	1033 ± 62

* k_3 values are average values from table (19)

$[E]_0 = 2.00x10^{-7}$ Mol L^{-1}

** C_3 - dl

\+ C_2 - dl

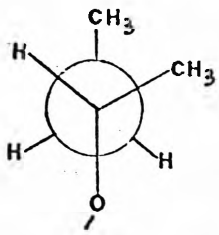
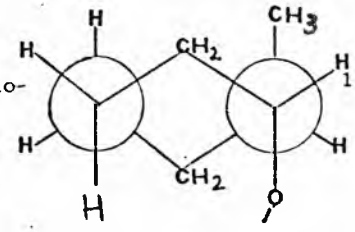
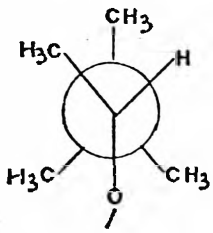
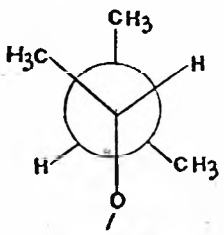
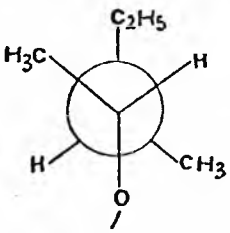
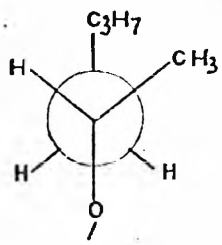
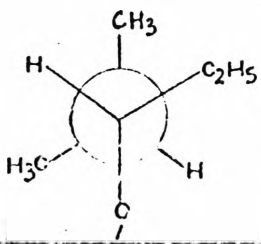
Table (19). Comparison of k_3 values obtained at two different initial substrate concentrations of L-phenylalanine (±)alkyl esters at 15°C

(±) R	k_3 values at $[S]_0'$ and $[S]_0''$	
	k_3' sec^{-1}	k_3'' sec^{-1}
(+) 2-butyl	0.90 ± 0.001	0.89 ± 0.05
(-) 2-butyl	1.19 ± 0.02	1.19 ± 0.09
(+) 2-methyl-cyclohexyl	2.49 ± 0.16	2.47 ± 0.34
(-) 2-methyl-cyclohexyl	1.95 ± 0.07	1.94 ± 0.15
(+) 3,3-dimethyl-2-butyl	1.51 ± 0.11	1.55 ± 0.15
(-) 3,3-dimethyl-2-butyl	0.83 ± 0.07	0.85 ± 0.14
(+) 3-methyl-2-butyl	1.73 ± 0.46	1.57 ± 0.65
(-) 3-methyl-2-butyl	0.61 ± 0.09	0.54 ± 0.16
(+) 3-methyl-2-pentyl	1.95 ± 0.06	1.89 ± 0.18
(-) 3-methyl-2-pentyl	1.67 ± 0.30	1.48 ± 0.53
(+) 4-methyl-2-pentyl	1.19 ± 0.19	1.11 ± 0.07
(-) 4-methyl-2-pentyl	2.75 ± 0.39	2.71 ± 0.25
(+) 2-methyl-3-pentyl	0.69 ± 0.02	0.66 ± 0.10
(-) 2-methyl-3-pentyl	0.64 ± 0.04	0.63 ± 0.07

$[E]_0$ = 2.00×10^{-7} Mol L^{-1}

pH = 7.5

Table (20) Newman projection formulae and K_m, k_3 for diastereoisomeric L-phenylalanine (±) alkyl esters.

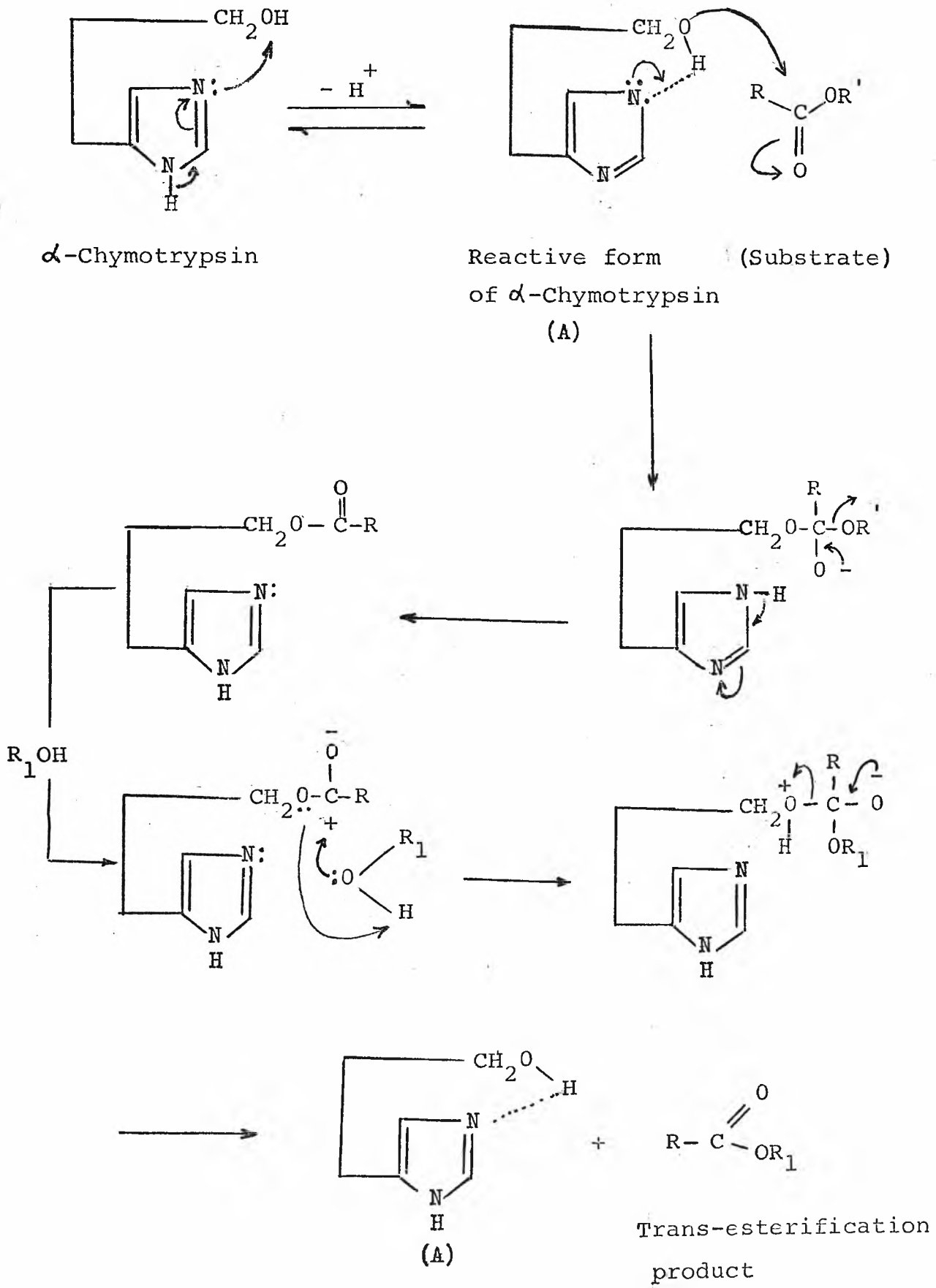
Ref. Table 18.	± R	Newman Projections	Diastereoisomer			
			K_m Mol L^{-1} X 10^{-3}		k_3 Sec^{-1}	
			(+)	(-)	(+)	(-)
a	2-butyl		0.73±0.12	0.67±0.10	0.90±.025	1.19±.09
b	2-methyl cyclo-hexyl		1.16±0.24	2.28±0.25	2.48±0.25	1.94±0.15
c	3,3'-dimethyl-2-butyl		1.40±0.16	2.10±0.35	1.53±0.13	0.83±0.11
d	3-methyl-2-butyl		2.37±1.02	1.10±0.51	1.65±0.56	0.56±0.12
e	3-methyl-2-pentyl		1.55±0.17	1.12±0.23	1.92±0.12	1.58±0.42
f	4-methyl-2-pentyl		1.18±0.26	1.32±.19	1.15±0.13	2.73±0.32
g	2-methyl-3-pentyl		0.32±0.17	0.62±.18	0.68±0.06	0.64±0.04

Kinetic Studies of α-Chymotrypsin in the Presence of Externally Added Nucleophiles:

It has been observed that when nucleophiles such as alcohols, amines etc. are added to the α-chymotrypsin catalyzed hydrolysis systems a trans-esterification reaction usually takes place resulting in a competitive partitioning of the acyl- enzyme intermediate by water and the added nucleophile (in our study alcohol was used). This may be interpreted in terms of a mechanism given in figure (19).

Bender et.al.[68] have postulated a similar reaction sequence.

Figure (19). Mechanism of trans-esterification reaction



Our study was mainly concerned with the isolation and the detection of the trans-esterification products. Typically L-phenylalanine (-) 2-octyl ester was subjected to α-chymotrypsin catalyzed hydrolysis in the presence of both (30% v/v) methanol and ethanol respectively and the course of hydrolysis was monitored by G. L. C. The intensity of the detector response for the trans-esterification products (methyl and the ethyl esters which were also confirmed by mass spectrometry, Figure (20)), revealed that the structure of the added nucleophile determines the rate of formation of the trans-esterification product and the overall hydrolysis rate of the substrate in general. Figure (21) gives a comparative study of the plots of the concentration of the unhydrolysed substrate against time for L-phenylalanine (-) 2-octyl ester in the presence and the absence of (30% v/v) added methanol and ethanol respectively. It will be observed from the plots that the initial rate of the reaction in the system free of any added nucleophile was decreased by a

factor of 1.4 when (30% v/v) methanol is added and the rate further decreases by a factor of almost 3.2 when (30% v/v) ethanol was added to the system.

Figure (20) M.S. of L-phenylalanine methylester (N-TFA derivative)

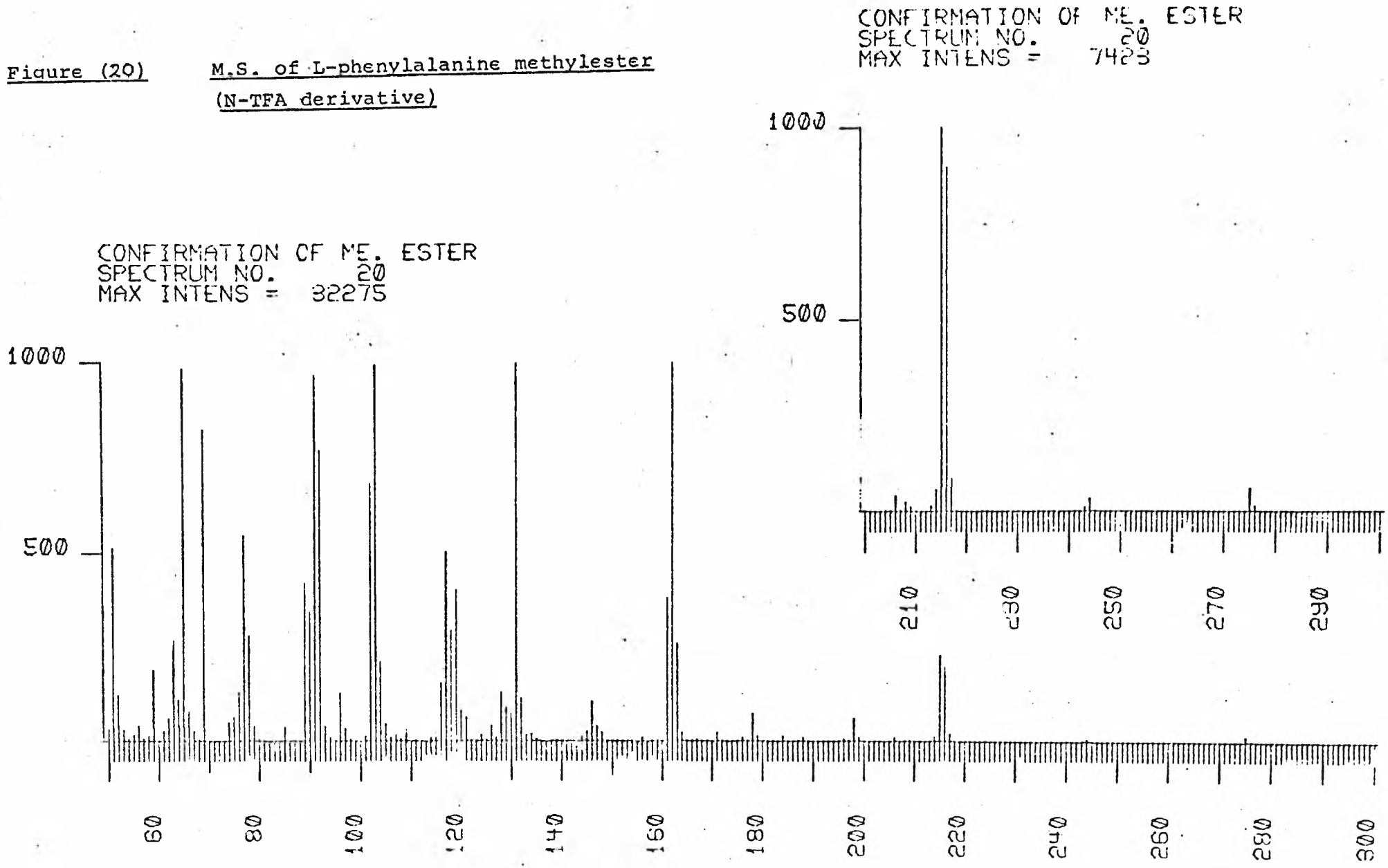
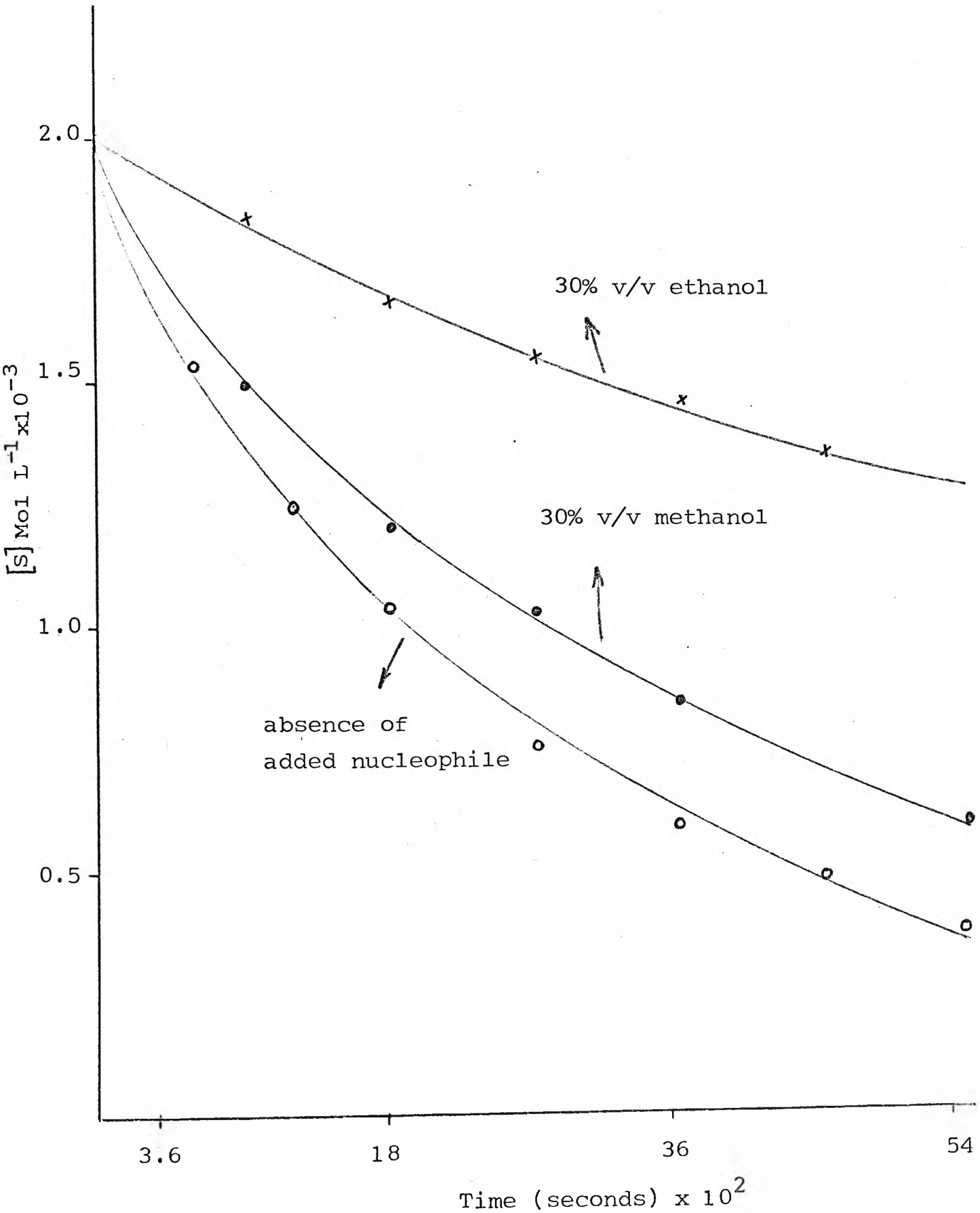


Figure (21). Effect of added nucleophile on L-phenylalanine (-)2-octyl ester hydrolysis



INHIBITION STUDIES

A substance which has the capability of decreasing the rate of a chemical reaction is called an inhibitor. We distinguish three kinds of inhibitor in enzymic reactions.

(i) Competitive Inhibitors

This type of inhibitor combines with the enzyme active sties thus partly blocking off the active sites to which the substrate binds itself during the formation of the enzyme-substrate complex.

(ii) Non-Competitive Inhibitors

The presence of a non-competitive inhibitor does not affect the combination of the substrate with the enzyme active site, but may affect the maximum velocity of the reaction by either combining with the enzyme-substrate complex or by allowing the enzyme-inhibitor complex EI to combine with the substrate in accordance with the scheme below,

$$E + S \rightleftharpoons ES$$

$$E + I \rightleftharpoons EI$$

$$ES + I \rightleftharpoons ESI$$

$$EI + S \rightleftharpoons EIS$$

(iii) Mixed Type Inhibitors

The third type of inhibitor is that which acts in both ways, namely by competing with the substrate for

the enzyme active site (as does the competitive inhibitor) and also by affecting the maximum velocity of the reaction (as does the non-competitive inhibitor). Such inhibitors are often referred to as uncompetitive inhibitors.[60]

Of these three kinds of inhibitor the competitive inhibitors have been most extensively studied. For these the enzyme-inhibitor equilibrium along with the enzyme-substrate equilibrium may be represented as :-

$$E + S \rightleftharpoons ES \longrightarrow E + \text{Product}$$

$$E + I \rightleftharpoons EI \quad \text{(where I represents the competitive inhibitor)}$$

It is assumed that there is no further decomposition of the enzyme-inhibitor complex. Compounds which are specially related to the substrate structure combine with the enzyme at the same enzyme active site as the substrate hence diminishing the combining power of the substrate and resulting in a change in the enzyme-substrate equilibrium relative to the enzyme-substrate equilibrium in the absence of the competitive inhibitor. Inhibition by the D-amino acid esters in a DL-amino acid ester mixture in the α-chymotrypsin catalyzed hydrolysis reaction has been shown to be competitive in most cases studied[8]. It has also been reported[10] that such competitive inhibitors react with the enzyme at the same enzyme active site as the normal substrate. In a DL-phenylalanine alkyl ester one half of the ester

(the D - amino acid moiety) acts as the inhibitor for the α-chymotrypsin reaction while the ester of the L-amino acid moiety undergoes the enzymic reaction as a normal substrate. Preliminary enzymic hydrolysis experiments using DL-phenylalanine alkyl esters revealed that the synthetic compounds contained approximately 1:1 mixture of D-and-L-phenylalanine alkyl esters. In these systems two enzymic equilibrium are normally anticipated; one the enzyme substrate equilibrium and the other enzyme inhibitor equilibrium, K_i (inhibitor constant) values of such systems may be evaluated using the equation (20) which has been derived below, whilst K_m and k_3 values in these systems may be determined by measuring the rate of change in the substrate concentration with time and then applying the extended integrated Michaelis-Menten equation 19, (page 80). This allowed us to examine the kinetic parameters of a substrate, in presence of the inhibitor and in the absence of the inhibitor thus to evaluate the effect of the inhibitor on the kinetics of the enzymic reaction. Table (21a) summarises our values along with the change in the free energy calaculated for both systems using the equation

$$\Delta(\Delta G^0) = -RT \ln K_m$$

Equation (20)

$$v = \frac{V_{max}\ [S]}{[S] + K_m\ (1 + \frac{[I]}{K_i})} \quad \text{.....Eq (20)}$$

may be derived as follows:

Rewriting the enzyme-substrate equilibrium in the presence of the competitive inhibitor, we have,

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + \text{Product} \quad \text{(Equilibrium 1)}$$

$$E + I \underset{k'_2}{\overset{k'_1}{\rightleftharpoons}} EI \quad \text{(Equilibrium 2)}$$

Applying the steady state Michaelis-Menten kinetics, we have for equilibirum 1 ,

$$k_1 [E] [S] - k_2 [ES] - k_3 [ES] = 0 \quad \ldots\ldots \text{Eq (21)}$$

but since part of the enzyme is also associated with the inhibitor in the form of EI we have,

$$[E] = [E_0] - [ES] - [EI] \quad \ldots\ldots\ldots \text{Eq (22)}$$

hence equation (21) becomes

$$k_1 [S] ([E_0] - [ES] - [EI]) = [ES] (k_2 + k_3) \quad \ldots\ldots \text{Eq (23)}$$

and for equilibrium 2,

$$k'_1 [I] [E] - k'_2 [EI] = 0 \quad \ldots \text{eq (24)}$$

or, substituting the value of $[E]$ from equation (22), equation (24) becomes,

$$k'_1 [I] ([E_0] - [ES] - [EI]) = k'_2 [EI] \quad \ldots\ldots \text{Eq (25)}$$

The over-all rate of the reaction is given by,

$$v = k_3 [ES]$$

Hence solving for v we have,

$$v = \frac{k_3 [E_0]}{1 + \frac{k_2 + k_3}{k_1} \cdot \frac{1}{[S]} \left(1 + \frac{k'_1}{k'_2} [I]\right)} = \frac{V_{max} [S]}{[S] + K_m \left(1 + \frac{I}{K_i}\right)}$$

where $K_i = \frac{k'_2}{k'_1}$

and K_m is Michaelis-Menten constant of the enzyme-substrate equilibrium in the presence of the inhibitor. The above equation (20) may be recasted into a linear form

$$\frac{1}{v} = \frac{K_m}{V_{max}} \left(1 + \frac{[I]}{K_i} \right) \cdot \frac{1}{[S]} + \frac{1}{V_{max}}$$

a plot of $\frac{1}{v}$ against $\frac{1}{[S]}$ yields a straight line with slope $\frac{K_m}{V_{max}} (1 + \frac{[I]}{K_i})$ and intercept as $\frac{1}{V_{max}}$

hence, $\frac{\text{slops}}{\text{intercept}} = K_m \left(1 + \frac{[I]}{K_i}\right)$

thus the value of K_i can be evaluated.

Since there is no break down of the enzyme-inhibitor complex into products the inhibitor constant K_i represents a true equilibrium constant. The affinity of an enzyme for two competitive inhibitors may therefore be compared by their respective K_i values but it is probably incorrect to compare the affinity of the enzyme for the substrate and the inhibitor by means of the K_m and K_i values. The free energy change for the formation of α-chymotrypsin-D-phenylalanine alkyl ester systems have been determined and are reported in Table (21). Observing the various parameters summarized in Table (14 & 21) it is seen that the K_m values for the phenylalanine alkyl ester substrates in the inhibited system have decreased by a factor of about 2.5 to 3.5 as compared to the values in the non-inhibited system. This indicates

a noticable influence of the competitive inhibitor on the enzyme-substrate equilibrium. A slight difference in the k_3 values are also observed which indicates that the over all catalytic activity of the enzyme is least affected by the presence of the competitive inhibitor. Better correlation in the k_3 values might be obtained by considering the rotational entropy factor as suggested before. $-\Delta(\Delta G^{O})$ values are also listed which are indicative of the degree of bonding between the enzyme and the substrate for both the systems.

The competitive inhibitors of α-chymotrypsin may be generalised in terms of a general formula $R_1CHR_2R_3$ where R_1,R_2 and R_3 are the three predominant structural features which determine the inhibition properties of the molecule. They are also mainly responsibile for combining with the specific groups in the enzyme active site to form the enzyme-inhibitor complex. Table(21) shows that in the general formula $R_1CHR_2R_3$, changes in R_3 (R_3= COOR, R has been varied) have been made in the synthetic compounds.

In general, increasing the bulk of the R_3 substituent increases the change in the free energy $-\Delta(\Delta G^{O})$ of formation of the enzyme-inhibitor complex. It is interesting to note that the introduction of chirality

in the R_3 substituent can often bring about considerable change in $-\Delta(\Delta G^{O})$. For example, in the cases of D-phenylalanine (+)2-octyl and D-phenylalanine(-)2-octyl esters a difference of 337 cal/mol in the $-\Delta(\Delta G^{O})$ value is obtained, Table (21). Another interesting observation can be made, regarding the characteristics of R_1. It will be noticed from $-\Delta(\Delta G^{O})$ values for compounds a,b and e,f (Table 21) that the introduction of electron withdrawing groups in the benzene ring of the D-phenylalanine results in an increased value for $-\Delta(\Delta G^{O})$.

Table (21) K_m, k_3, K_i and $-\Delta(\Delta G^o)$ values for α-Chymotrypsin catalyzed hydrolysis of DL-phenylalanine alkyl esters at 15°C.

	Inhibitor * (D-aminoacid ester) R	K_i Mol L^{-1} x10^{-3}	$-\Delta(\Delta G^o)$ cal Mol^{-1} x	DL-substrate	
				K_m Mol L^{-1} x10^{-3}	k_3 sec^{-1}
a	n-butyl	0.85±0.20	4046±121	0.87±0.15	6.27±0.54
b	(-)2-methyl-1-butyl	0.29±0.25	4661±355	0.61±0.22	4.00±1.1
c	(-)2-octyl	0.126±0.02	5138±84	0.34±0.05	1.73±0.12
d	(+)2-octyl	0.07±0.004	5475±32	0.21±0.04	1.73±0.20
e	n-butyl**	0.40±0.07	4477±92	0.14±0.03	1.76±0.08
f	(-)2-methyl-1-butyl**	0.17±0.06	4967±173	0.12±0.1	2.13±0.42

Experimental Conditions

pH = 7.5

$[E]_0 = 2.00 \times 10^{-7}$ Mol L^{-1}

* $[I]_0 = 1.00 \times 10^{-3}$ Mol L^{-1}

$[S]_0 = 1.00 \times 10^{-3}$ Mol L^{-1}

** p-fluoro-D-phenylalanine-COOR

x $-\Delta(\Delta G^o)$ refers to the degree of bonding between the enzyme and the inhibitor.

Table (21a) Comparison of Kinetic parameters in α-chymotrypsin-phenylalanine alkyl ester system in the presence and absence of inhibitor.

R	INHIBITED SYSTEM			UNINHIBITED SYSTEM		
	K_m Mol L^{-1} x10^{-3}	k_3 Sec^{-1}	ΔG Cal/mol	K_m Mol L^{-1} x10^{-3}	k_3 Sec^{-1}	ΔG Cal/mol
n-butyl	.87±.15	6.27± .54	4032± 91	2.19± .022	7.17± .85	3492±41
(-)2-methyl -1-butyl	.61±.22	4.00±1.10	4235±176	1.62± .22	4.29± .20	3664±73
(-)2-octyl	.34±.05	1.73± .12	4570± 79	.85± .06	2.39± .04	4031±39
(+)2-octyl	.21±.04	1.73± .20	4846±100	.71± .04	2.13±.025	4134±32
n-butyl**	.14±.03	1.76± .08	5078±111	-	-	-
(-)2-methyl** -1-butyl	.12±.10	2.13± .42	5166±347	-	-	-

** (Cf Table 21)

- EXPERIMENTAL -

GENERAL:

All the alcohols used in this work were obtained from commercial sources in a pure or analytical grade. They were distilled and in some cases had to be fractionated before use. The alcohols were obtained from Fluka A.G. (Switzerland) with the following exceptions; n-butanol from E.Merck A.G. Darmstadt (Germany), sec-butanol from Unilab (Sydney) and n-octanol from B.D.H. Chemicals (Poole, U.K.). All amino acids were obtained from B.D.H. Chemicals (Poole, U.K.) except p-fluoro and p-nitro DL-phenylalanine which were supplied by Mann Research Laboratory (U.S.A.). p-Toluene sulphonic acid was obtained from E.Merck A.G.Darmstadt (Germany).

The α-chymotrypsin used in this work was three times crystallised, lyophilized grade and was obtained from bovine pancreas. Its activity was 45 units/mg solid, and it was supplied by Sigma Chemicals (U.S.A.) lot number 92c-8120.

Melting points of the substrates were

recorded with a Gallenkemp melting point apparatus, and are uncorrected. Thin layer plates were prepared using Merck Kieselgel G nach stahl.

Gas chromatographic analyses were carried out on a Becker 409 Gas chromatograph fitted with a hydrogen flame ionization detector. The gas chromatographic analyses (G.L.C.) were run on a 6'x1/8" stainless steel column packed with O.V. 17 (5%) on Chromosorb-W, or DEGS/EGS-X(0.7/0.3 %w/w) on dimethyldichlorosilane (D.M.C.S.) treated Chromosorb-W. The nitrogen flow was 25 ml/min during the analyses.

The chemical identities of the substrates were confirmed by G.L.C. and mass spectrometry figure (22) using a Varian model 600D Gas Chromatograph coupled to a E.A.I. Quadropole Mass Spectrometer via a Biemann Separator. The mass spectrometer was run with a 70 electron volt energy beam and during the analyses the source temperature was maintained at 200^{o}C.

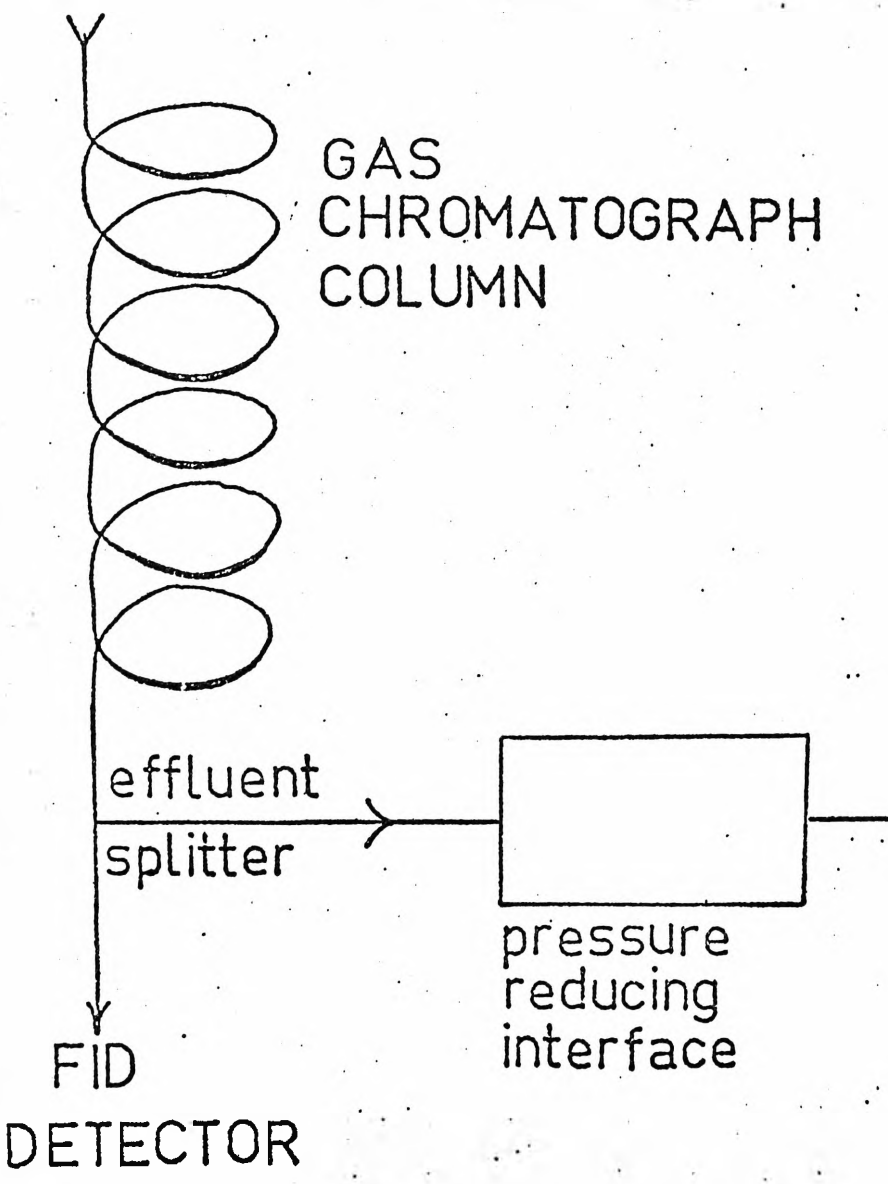
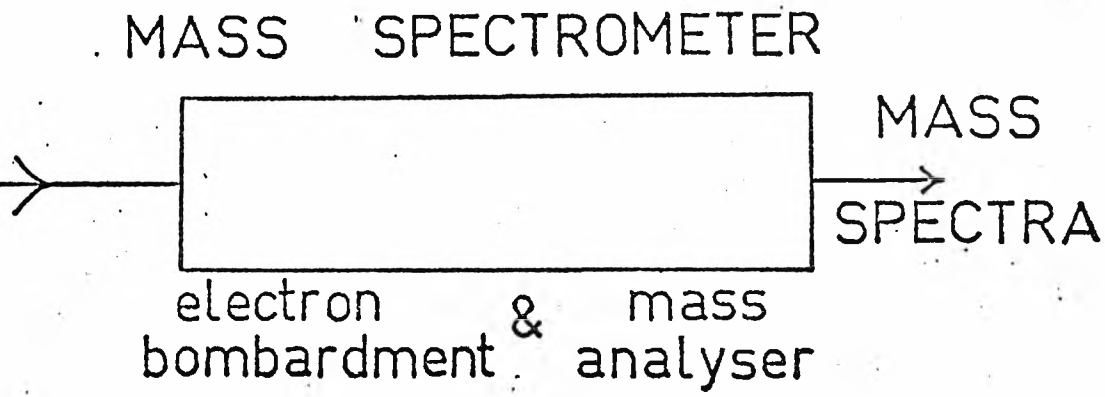

GAS
CHROMATOGRAPH
COLUMN
effluent
splitter
pressure
reducing
interface
FID
DETECTOR

Figure (22) Flow-Chart of G.L.C -M.S. Technique



SYNTHESIS OF SUBSTRATES

All alcohols were distilled before esterification and all solvents used were dried and kept over molecular sieve 3A.

Section (1) : Preparation of L-phenylalanine (±) alkyl ester p-toluenesulphonates

L-phenylalanine (0.025M; 4.13 gm), alcohol (0.058M) from Table (22) and p-toluenesulphonic acid (0.035M; 6.0gm) were added to a mixture of benzene (35ml) and toluene (15ml) and the suspension was refluxed with vigorous stirring. The water formed during the esterification process was removed by means of a Dean and Stark head. The reaction time required for complete esterification varied between 10 to 30 hours depending on the alcohol used.

After the esterification was complete, the reaction mixture was cooled and filtered to remove traces of unreacted L-phenylalanine and

Table (22). List of alcohols and amounts used for the esterification of L-phenylalanine

Alcohols	molecular weight	weights, corresponding to 0.058M (gm)
2-methyl-1-butanol	88	5.10
2-butanol	74	4.30
3-methyl-2-butanol	88	5.10
3,3-dimethyl-2-butanol	102	5.90
4-methyl-2-pentanol	102	5.90
3-methyl-2-pentanol	102	5.90
2-methyl-3-pentanol	102	5.90
2-methyl cyclohexanol	114	6.60
menthol	156	9.10
methanol	32	1.85
ethanol	46	2.70
n-propanol	60	3.48
n-butanol	74	4.30
n-amyl alcohol	88	5.10
n-octanol	130	7.50
cyclohexanol	100	5.80
1-cyclohexyl ethanol	128	7.43
2-octanol	130	7.50

p-toluenesulphonic acid. The solvent was then evaporated at 40°C in a rotary evaporator. The oily or partially crystalline residue thus obtained was triturated with anhydrous ether and the mixture was then allowed to stand at room temperature for a few hours. In some cases it was necessary to cool the mixture to 0°C overnight before complete solidification of the substrate took place. The product was then collected by filtration and then dried under vacuum . It was stored in a dessicator over silica gel. Attempts to purify the L-phenylalanine (±) alkyl ester p-toluenesulphonates by fractional crytallization resulted in partial or even complete separation of the diastereorisomeric mixture. This made further purification of the diastereoisomeric ester substrates not possible. The diastereoisomeric composition of the alkyl ester substrates were determined by G.L.C.Table (23) of their N-trifluoroacetyl derivatives.

Table (23). Physical constants of L-phenylalanine (±) alkyl ester p-toluenesulphonates

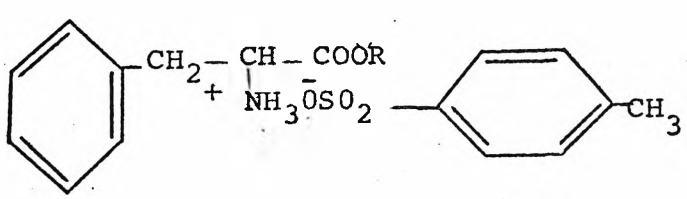
R+	M.P. °C	Diastereoisomeric Composition ** L(+)/L(-)	Retention Time (min) L(+)	L(-)
2-methyl-1-butyl*	114.5-116	1.00	7.70	7.70
2-butyl	117.5-119	1.22	8.47	7.92
3-methyl-2-butyl	95 - 97	1.08	9.25	8.62
3,3-dimethyl-2-butyl	134 -135	1.50	10.75	9.92
4-methyl-2-pentyl	97 - 98	1.22	9.25	8.85
3-methyl-2-pentyl	106 -107	1.38	10.75	10.08
2-methyl-3-pentyl	113 -114	1.17	10.33	9.70
2-methyl cyclohexyl	128 -130	1.22	15.84	15.20
menthyl	126 -130	1.00	18.38	16.70

* Not resolved.

G.L.C. run on 6'x1/8" stainless steel column packed with 5% O.V.17 on Chromosorb-W. Temperature programme 150 - 250 °C at 10 °C/min.; Nitrogen flow 25 ml/min.

** G.L.C. Condition : 6'x1/8" stainless steel column packed with 0.7/0.3 % w/w DEGS/EGS-X on D.M.C.S. treated Chromosorb-W. Temperature programme 150 - 200 °C at 4 °C/min.; Nitrogen flow 25ml/min.

+General Structure :



Section (2) : Optical Resolution of Asymmetric Alcohols

(a) Preparation of Optically pure L-valine (+) alkyl ester:

Asymmetric alcohols were conveniently resolved via their L-valine alkyl esters. These were synthesised as follows:

L-valine (0.025M: 2.92g), the (±) alcohol (0.058M) and p-toluenesulphonic acid (0.035M: 6.0gm) were refluxed in a mixture of benzene (35 ml) and toluene (15ml) using a Dean-Stark head as described in section (1). After removal of the solvent, the mixture containing the diastereosmeric L-valine (±)alkyl ester p-toluenesulphonate was diluted with anhydrous ether and the crude product was filtered and washed with ether. After several recrystalisation from benzene the L-valine (+) alkyl ester p-toluenesulphonate was obtained in about 98% optically pure form. The optical purity of the L-valine alkyl esters was determined by G.L.C. analysis of the N-TFA derivatives.

G.L.C. Analysis of N-TFA - L-valine (+) alkyl esters

The N-TFA derivatives were prepared by dissolving the L-valine (+) alkyl ester p-toluene-sulphonates (1-2 mg) in ethyl acetate (0.5ml) and trifluoroacetic anhydride (0.1ml). The reaction mixture was then allowed to stand at room temperature for about one hour, after which the solvent and excess reagent were removed. The residue was then dissolved in ethyl acetate (0.5ml) and the organic layer was washed with water (1.0ml) and the solution was dried (anhydrous Na_2SO_4).
A sample (2 μl) was injected in the G.L. C. column Table (24).

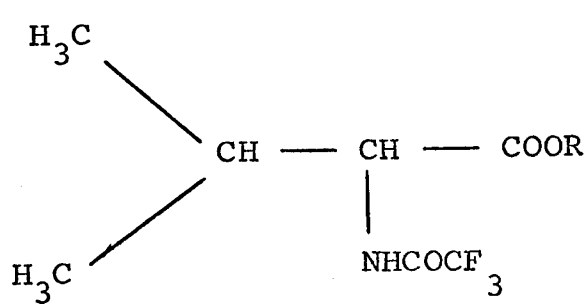
Table (24). G.L.C. of N-TFA-L-valine(+)alkyl esters

R†	M.P. °C*	Optical Purity	Retention Time (min)**
4 methyl-2-pentyl	165°	95%	10.85
2-methyl cyclohexyl	181°	98%	18.35
3-methyl-2-butyl	154-156°	99%	10.80
3,3-dimethyl-2-butyl	185°-187°	100%	11.95
3-methyl-2-pentyl	121°-122°	97%	12.00

* M.P's of the alkyl p-toluenesulphonates

** G.L.C. Condition : 6' x1/8" stainless steel column packed with 0.7/0.3% w/w DEGS/EGS-x on Chromosorb-W (D.M.C.S. treated). Temperature programme 80° -200°C at 4°C/min. Nitrogen flow 25 ml/min.

\+ General Structure



(b) Preparation of Optically Pure (+) sec-alcohols from L-valine (+) alkyl ester:

The Optically pure L-valine (+) alkyl ester p-toluenesulphonates (0.01M) from section(2a)were hydrolyzed with an aqueous solution of sodium hydroxide (0.02M) at room temperature. The course of the hydrolysis was followed by T.L.C. using the solvent system (butanol : acetic acid : water :: 70 : 15 : 15), and the products were visualized with ninhydrin spray. The reaction was stopped after the faster moving ninhydrin positive spot had disappeared. The (+) alcohol was then extracted into ether and the dried extract was fractionally distilled at atmospheric pressure to give the optically pure (+) alcohols.

Section (3a) Preparation of L-phenylalanine(+) alkyl p-toluenesulphonate

Small amounts of optically active pure (+)sec-alcohols were made from L-valine (+)alkyl p-toluenesulphonates for characterization purposes.

The L-phenylalanine (+)alkyl p-toluenesulphonates were conveniently prepared from the ether extracts of the (+)alcohols (Cf.Section 2 b) by refluxing in the presence of L-phenylalanine and p-toluenesulphonic acid using Dean and Stark head (Cf.Section 1). The products were analysed gas chromatographically via their N-TFA derivatives (Table 25).

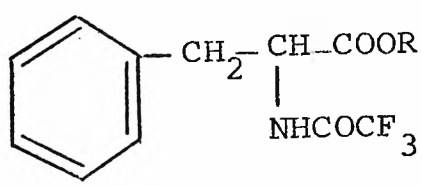
Table (25). G.L.C. of N-TFA L-phenylalanine (+) alkyl ester

R+	M.P. °C *	Optical purity	Retention Time (min) **
4-methyl-2-pentyl	147-148	98%	9.25
2-methyl cyclohexyl	167-168	98%	15.84
3-methyl-2-butyl	113-115	100%	9.25
3,3-dimethyl-2-butyl	129-130	100%	10.75
3-methyl-2-pentyl	152-153	95%	10.75

* Melting points are of the L-phenylalanine (+) alkyl p-toluenesulphonates.

** G.L.C. analysis : 6' x 1/8" stainless steel column packed with 0.7/0.3 % w/w DEGS/EGS-X on D.M.C.S. treated Chromosorb-W. Temperature programme 150-200 °C at 4 °C/min.; Nitrogen flow 25ml/min.

\+ General Structure



Section (3b) Preparation of Optically Pure L-phenylalanine (+) or (-)alkyl ester p-toluenesulphonates.

L-phenylalanine (0.025M; 4.1gm), the optically active alcohol (0.058M; Table 23) and p-toluenesulphonic acid (0.035M; 6.0gm) were refluxed in a mixture of benzene (35ml) and toluene (15ml) as described in Section (1). The compounds were analyzed for optical purity by G.L.C. analysis of the N-TFA derivatives (Table 26).

Table (26). G.L.C. of optically pure N-TFA L-phenylalanine (+) or (-) alkyl esters.

R[+]	M.P. C[*]	Retention time[**] (min)
(-) 2-methyl-1-butyl	116-118	7.33
(-) 2-octyl	75-79	9.85
(+) 2-octyl	119-122	9.90
(+) 1-cyclohexyl ethyl	-	10.50

* p-toluenesulphonate salts.

** G.L.C. analysis : 6' x 1/8" stainless steel column packed with 5% 0.V.17 on Chemosorb-W at 10°C/min. Temperature programme 150° - 250°C. Nitrogen flow 25ml/min.

\+ General Structure. (Cf Table 25)

Section (4): Preparation of L-phenylalanine alkyl ester p-toluenesulphonate (using optically inactive alcohols.

The following alcohols listed in Table(27) were used to prepare L-phenylalanine ester substrates by the method described earlier in Section (1).

The substrates were analysed for chemical purity by G.L.C. of their N-TFA derivatives Table (28).

Table (27). List of Optically inactive alcohols used for the esterification of L-phenylalanines

Alcohols	B.P. °C	M. W.
n-butanol	117	74
n-octanol	195	130
n-propanol	98	60
amyl	138	88
ethanol	78	46
methanol	65	32
cyclohexanol	161	100

Table (29). Physical constants of L-phenylalanine alkyl p-toluenesulphonates.

R	M.P. °C	Retention time of the N-TFA derivative min*
n-butyl	124 - 125	7.10
n-octyl	131 - 132.5	11.20
n-propyl	167.5	4.90
amyl	138 - 139	7.50
ethyl	137.5-138	5.55
methyl	156 - 157**	4.85

* G.L.C. analysis : 6' x 1/8" stainless steel column packed with 5% O.V.17 on Chromosorb-W. Temperature programme 150° - 250° C at 10° C/min.; Nitrogen flow 25ml/min.

** HCl salt.

Section (5) : Preparation of racemic phenylalanine and substituted DL phenylalanine (+) and (-) alkyl ester p-toluenesulphonates.

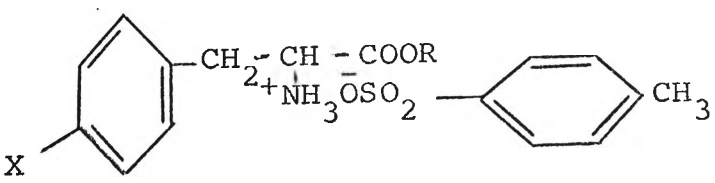
The physical properties of the substrates prepared from DL-phenylalanine , **p-fluoro** - DL-phenylalanine and **p-nitro-** DL-phenylalanine using optically inactive and optically pure alcohols are listed in Table (29). These compounds were prepared by the procedure outlined in section (1).

Table (29). Physical properties of DL-phenylalanine and substituted DL-phenylalanine (+) and (-) alkyl ester p-toluenensulphonates

X	R+	M.P.C°	Retention time of N-TFA derivative (min)*
H	n-butyl	120.5	6.90
"	(-) 2-octyl	66 - 67	9.85
"	(-) 2-methyl-1-butyl	121-122	7.40
"	n-octyl	116	9.80
"	(+) 2-octyl	-	9.85
F	n-butyl	dec 170	6.65
"	(-) 2-methyl-1-butyl	111 -113	7.15
"	(-) 2-octyl	182(dec)	9.60
NO_2	n-butyl	dec 180	6.60
"	(-) 2-methyl-1-butyl	138-140	6.90
"	(-) 2-octyl	-	9.55

* G.L.C. were run on a 6'x1/8" stainless steel column packed with 5% O.V.17 on D.M.C.S.treated Chromosorb-W. Temperature was programmed from 150°-250°C and the Nitrogen flow was 25ml/min.

+ General Structure



Enzymic Hydrolysis:

Enzyme hydrolyses were carried out in a thermostatically controlled water bath, in which temperature control was maintained by means of an immersion heater which was connected to a Beckman contact thermometer set at the desired temperature. Cooling was achieved by means of a refrigerated probe which was immersed in the water bath, and its controls were synchronised with the heater and the Beckman thermometer. The temperature fluctuations in this set up were less than $0.2^{o}C$. To minimise the evaporation losses, small plastic balls were used to cover the surface of the water bath.

Enzyme Solutions: The enzyme solution was made up just before each hydrolysis experiment. The preparation of α-chymotrypsin solutions of known concentration were based on a molecular weight of 24,800 of the enzyme[17]. The exact enzyme concentrations were then determined

spectrophotometrically by measuring the optical density of the solution at 282 mμ . It was found that the enzyme solutions could be stored in a refrigerator at 0°C up to one week period during which the strength of the solution was constantly checked spectrophotometrically.

Substrate Solutions: Substrate solutions were prepared by dissolving the esters in the buffer at pH 7.5. The buffer was prepared by adjusting the pH of an aqueous solution of analytical grade tris-(hydroxymethyl)- aminomethane (T.H.A.M.) 0.25M to 7.6 - 7.7 with 1N hydrochloric acid and diluting the resulting solution with distilled deionised water (1:10) dilution.

Enzymic Hydrolysis: The substrate solutions of desired molarity were prepared by dissolving the substrates (cf.Table 30 for molarity to substrate weight relationship) in the buffer (20 ml) at pH 7.5 ± .05. An aliquot (10 ml) was then placed in the water bath and the system was allowed to

Table (30). Molarity to weight relationship of the substrates

X	R^+	M.W.	Weights corresponding to the molarities (mg/20ml solution) 0.002M	0.0055M
H	methyl	215*	8.60	23.65
"	ethyl	365	14.60	40.15
"	n-propyl	379	15.16	41.69
"	n-butyl	393	15.72	43.23
"	2-butyl	393	15.72	43.23
"	n-amyl	407	16.28	44.77
"	n-cotyl	449	17.96	49.39
"	2-octyl	449	17.96	49.39
"	2-methyl-1-butyl	407	15.72	44.77
"	3-methyl-2-butyl	407	15.72	44.77
"	3-methyl-2-pentyl	421	16.84	46.31
"	4-methyl-2-pentyl	421	16.84	46.31
"	2-methyl-3-pentyl	421	16.84	46.31
"	3,3'-dimethyl-2-butyl	421	16.84	46.31
"	cyclohexyl	421	16.84	46.31
"	1-cyclohexyl ethyl	311*	12.44	34.21
"	2-methyl cyclohexyl	428	17.12	47.08
"	menthyl	476	19.04	52.36
F	n-butyl	411	16.44	45.21
"	2-methyl-1-butyl	425	17.00	46.75
NO_2	n-butyl	438	17.52	48.18
"	2-methyl-1-butyl	452	18.08	49.72

* hydrochloride salt

\+ General Structure. (Cf Table (29)

come to equilibrium. In a similar fashion the enzyme solution was placed in the water bath to reach the same temperature. At zero time the enzyme solution (0.1 ml) was added to the substrate solution (10ml) and the resulting solution was shaken vigorously to achieve instantaneous mixing.

Samples (1.0ml) were withdrawn at regular intervals for analysis. The substrate concentration of these samples were determined by treating the sample solution with sodium bicarbonate (10% Aq, 1ml) and an internal standard solution (0.1 to 0.2ml) and extracting the resulting mixture with chloroform (3x10ml). The combined chloroform extract was then dried (anhydrous $MgSO_4$) and the solvent was evaporated keeping the temperature below $40^{o}C$ by use of a rotary evaporator. The oily residue was then derivatised for G.L.C. by adding trifluoroacetic anhydride (0.1 - 0.15 ml) at room temperature. After one hour the excess

reagent was evaporated off and the residue was dissolved in ethyl acetate (1ml). The ethyl acetate solution was then washed with water and dried. A sample ($\simeq 2\mu l$) was then injected into the gas chromatograph and the peak heights of the substrate and the internal standard peaks were measured. The substrate concentration of the samples thus determined were plotted against time.

In all cases substrate blanks without the addition of the enzyme were run to determine the extent of non enzymic hydrolysis at the appropriate temperature and pH.

Non enzymic hydrolysis for the substrates used in this work under the experimental time limit (not more than 180 minutes) was negligible.

Enzymic resolution of (±)alcohols via L-phenylalanine (±)alkyl esters

Phenylalanine (±)alkyl p-toluenesulphonate (1gm) was dissolved in T.H.A.M. buffer (100ml) and the pH was adjusted to 7.5. α-Chymotrypsin solution (0.1ml, concentration equivalent to 0.25mg/ ml reaction volume) was added and the reaction mixture was clamped at room temperature over a magnetic stirrer. Samples (0.5ml) were drawn initially at an interval of 15 minutes (after which the sampling time was varied according to the progress of the reaction) and extracted into cold chloroform (4x5ml) containing aq. sodium bicarbonate solution (1 ml, 20%) and a suitable internal standard. The chloroform layer was dried over anhydrous $MgSO_4$ and the solvent was evaporated at 40°C using a rotary evaporator. The unhydrolyzed ester was derivatised using trifluoro acetic anhydride and sample analyzed gas chromatographically.

The reaction was stopped when most of the more reactive isomer had been hydrolyzed. The unhydrolyzed ester was extracted into chloroform (4x25ml) containing aqueous $NaHCO_3$ (5ml, 20%). On removal of the solvent

the phenylalanine alkyl ester was obtained as an oily liquid which was converted to the p-toluene sulphonate salt by refluxing in anhydrous benzene containing p-toluene sulphonic acid and using Dean-Stark head. After refluxing for 2-Hrs the mixture was cooled and filtered to remove excess p-toluene sulphonic acid. The benzene solution on concentration yielded a crystalline solid on cooling. On recrystallization (twice) from benzene the product was obtained in 95-100% optically pure (G.L.C. analysis of the N-TFA derivative) form. Approximate yields are recorded in the table (31).

The optically pure esters thus obtained may be hydrolyzed to yield optically pure alcohols (43).

Table (31). Enzymic resolution of L-phenylalanine (±)alkyl esters

(±) R	G.L.C.Analysis* Isomer Recovered	Optical Purity	Yield**
2-methyl Cyclohexyl	L(-)	99%	15%
3,3-dimethyl-2-butyl	L(-)	95%	5%
3-methyl-2-butyl	L(-)	95%	low
menthyl***	-	-	-
4-methyl-2-pentyl	L(+)	98%	5%

* G.L.C. analysis were run using 6' x1/8" stainless steel tube packed with D.M.C.S treated DEGS/EGS-X (0.7/0.3%w/w) on Chromosorb-W. Nitrogen flow was 25ml/ml and temperature programme was maintained at 150°-200°C at 4°C/min.

** yields are recorded as the % of the starting amount used.

*** could not be followed quantitatively due to solubility problem.

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****

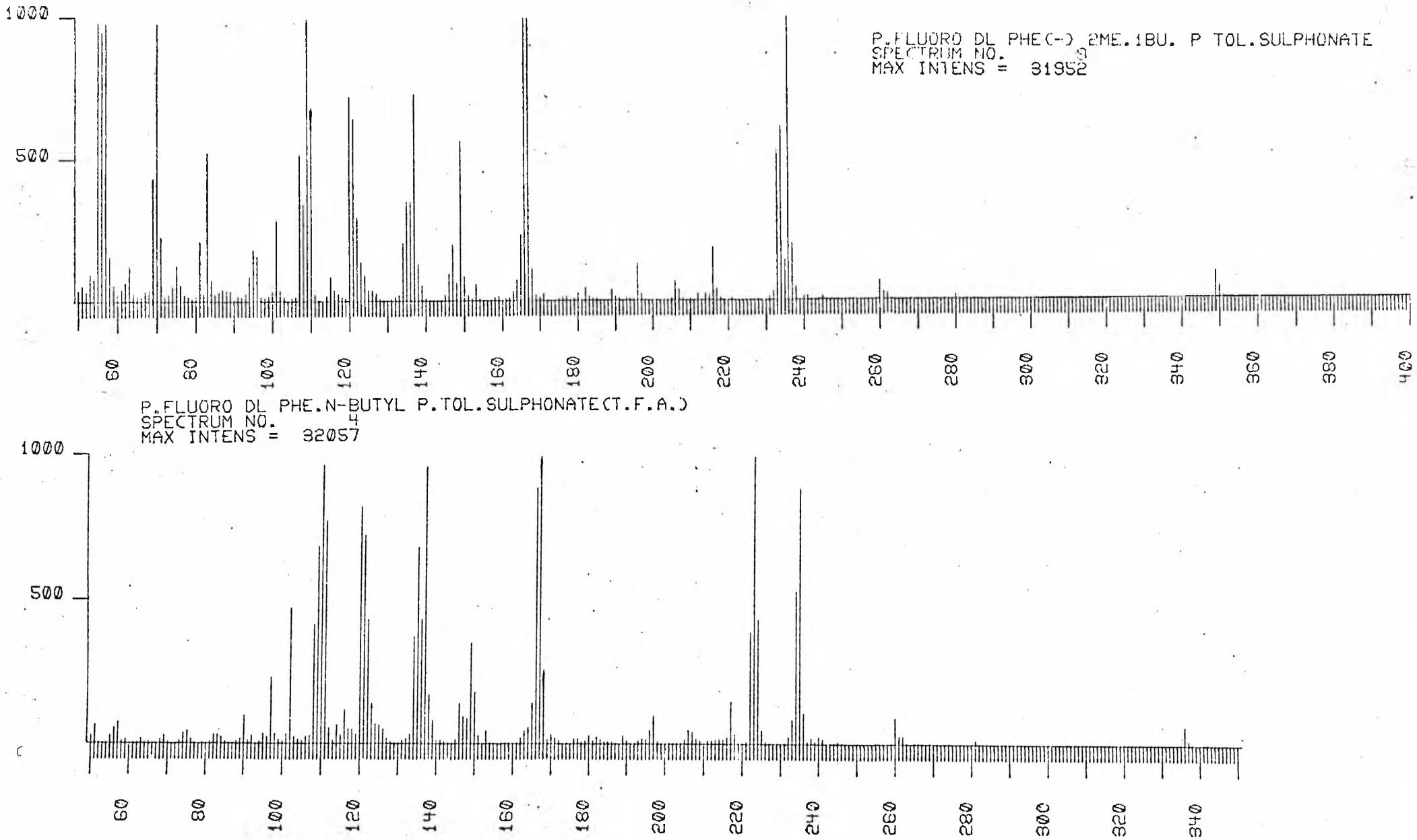
****

-APPENDIX-

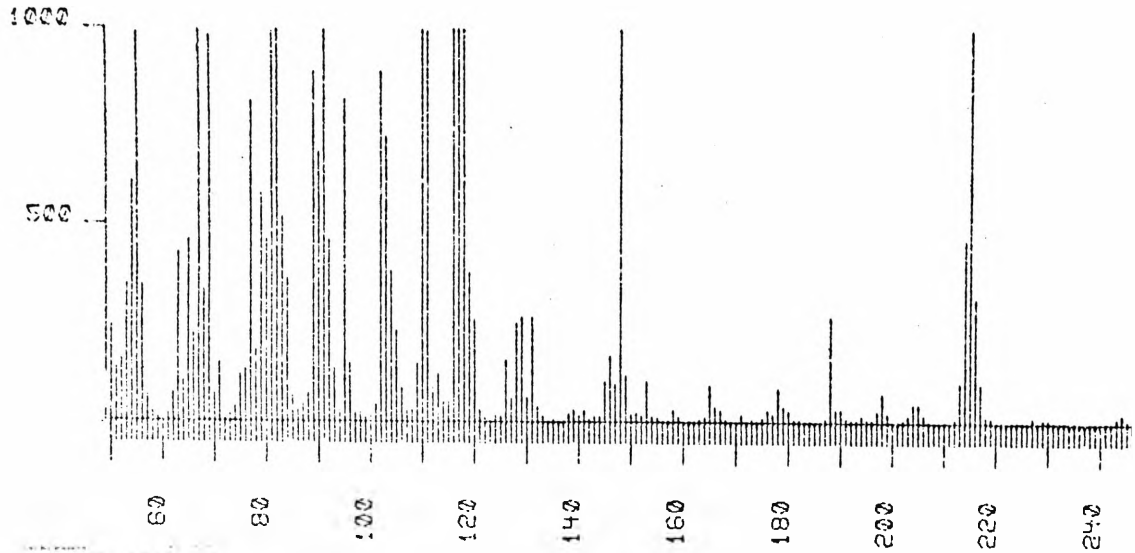
Mass spectrometry of phenylalanine alkyl esters have been discussed in detail in the discussion section (p.39); the following mass spectra are representative of the N-TFA-phenylalanine alkyl esters which have been characterized for structural identifications.

P-NITRO DL PHE(-)2ME.1BU.P.TOL.SULPHONATE(TFA)
SPECTRUM NO. 8
MAX INTENS = 2857

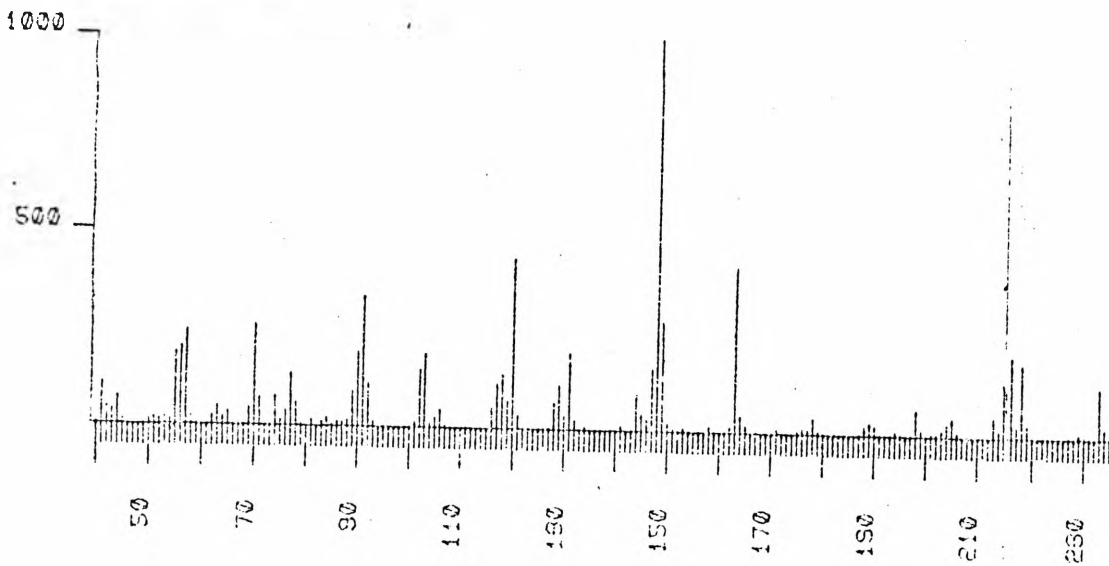
P-NITRO DL PHE(-)2ME.1BU.P.TOL.SULPHONATE(TFA)
SPECTRUM NO. 8
MAX INTENS = 17857


P.FLUORO DL PHE(-) 2ME.1BU. P TOL.SULPHONATE
SPECTRUM NO. 3
MAX INTENS = 31952
1000
500
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
360
380
400
P.FLUORO DL PHE.N-BUTYL P.TOL.SULPHONATE(T.F.A.)
SPECTRUM NO. 4
MAX INTENS = 32057
1000
500
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340

L PHE(+-)CYCLOHEXYL ETHYL P.TOL.SULPHONATE(TFA) 18 FEB. 1974
SPECTRUM NO. 30
MAX INTENS = 31854

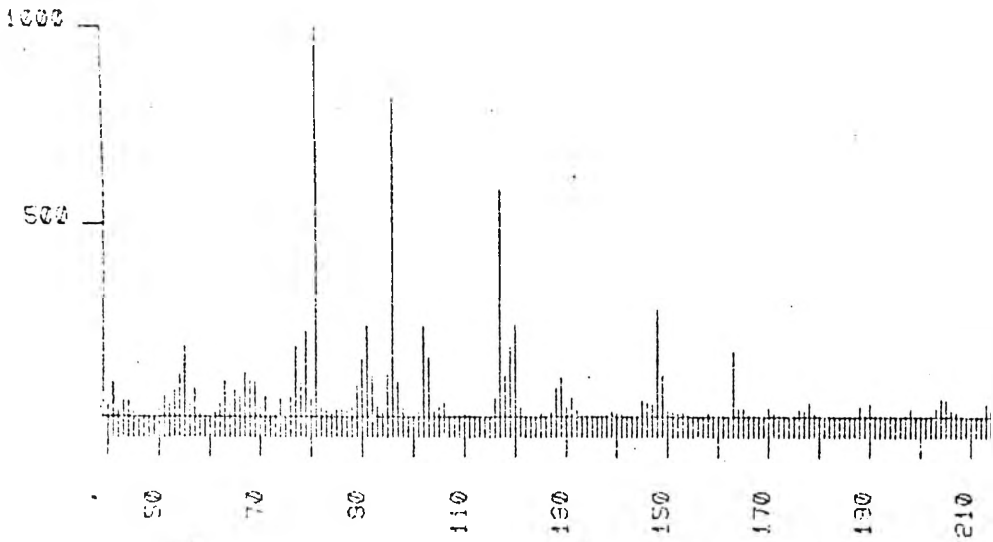


L PHE( ) 2NE 1BUTYL P.TOL.SULF. (T.F.A.)
SPECTRUM NO. 4
MAX INTENS = 15861

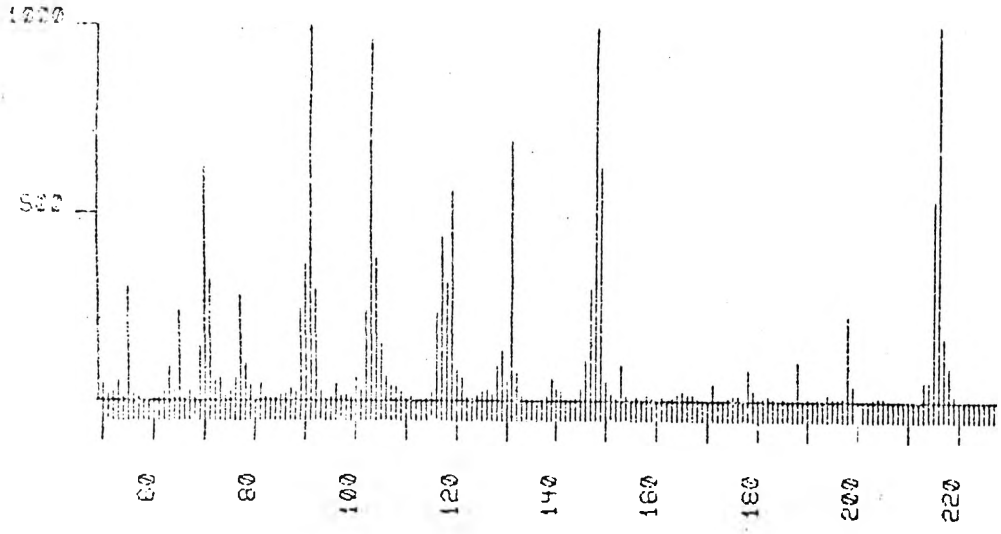


250
270
290
310
330
350
M+
250
280
300
320
340
360
380
400

L PHE( ) 2ME CYCLOHEXYL P.TOL.SULF.(T.F.A.)
SPECTRUM NO. 9
MAX INTENS = 29174



L PHE(+-) S ME.2BU. P.TOL.SULPHONATE(T.F.A.)
SPECTRUM NO. 2
MAX INTENS = 31981



240
260
280
300
320
340
M+
290
250
270
290
310
330
350
370
390

COMPUTER PROGRAMMES

Computer programmes were used to perform the more complicated calculations. Four programmes were written iin simple FORTRAN language.

PROGRAMME 1 (Main 1) calculates K_m and k_3 using the integrated Michaelis-Menten equation in the linear form.

$$\frac{1}{t} \ln \frac{[S]_0}{[S]} = \frac{k_3}{K_m} [E]_0 - \frac{1}{K_m} \frac{[S]_0 - [S]}{t}$$

whereby, from the slope and intercept both k_3 and K_m may be calculated.

PROGRAMME 2 (Main 2) determines the inhibition constant for α-chymotrypsin catalyzed hydrolysis in presence of the D-phenylalanine alkyl ester.

$$\frac{1}{v} = \frac{K_m}{V_{max}} \left(1 + \frac{[I]_0}{K_i}\right) \cdot \frac{1}{[S]} + \frac{1}{V_{max}}$$

$$\text{Slope/Intercept} = K_m \left(1 + \frac{[I]_0}{K_i}\right)$$

hence, the value of K_i may be calculated using known values of K_m and $[I]_0$.

PROGRAMME 3 (Main 3) was used to calculate the K_m and k_3 values using the equation

$$([S]' - [S]'') = K_m \left(\ln \frac{[S]_0'}{[S]'} - \ln \frac{[S]_0''}{[S]''}\right) + \left([S]_0' - [S]_0''\right)$$

the value of K_m thus determined was then substituted in eq. (17 or 18 p.80) to obtain the value of k_3. The programme Main 3 contains a statement

```
const = .0000002
```

which refers to the enzyme concentration and must be replaced by the enzyme concentration in use.

Programmes main 1 and 3 incorporates the subroutine WTDLS (weighted least squares) which performs a weighted linear least squares regression analysis of the equation

$$Y = A.X + B.$$

WTDLS may also be used without using weighted least square by substituting KK = 1.

An allowance of $\pm 4\%$ (estimated experimental error) was incorporated in the programmes to evaluate the upper and the lower limits of the concentrations from which standard error estimations were calculated.

The programmes and the explanatory flow chart are as follows.

```
1 $E 8*UPLI.FORFLO,S J,I
2 FLOWCHARTED BY  FORFLO /X809/ ON 13 NOV 76 AT 15:29:51
3
4
5
6
7  000001.           SUBROUTINE WTOLS (W,X,Y,N,SLOPE,YINT,SDS,SDINT,SDFIT,VSUM,CORR)
8  000002.           DIMENSION X(N),Y(N),W(N)
9  000003.           WW=0.0
10 000004.           WX=0.0
11 000005.           WY=0.0
12 000006.           WXX=0.0
13 000007.           WYY=0.0
14 000008.           WXY=0.0
15 000009.           DO 777 I=1,N
16 000010.           WW=WW+W(I)
17 000011.           WX=WX+X(I)*W(I)
18 000012.           WY=WY+Y(I)*W(I)
19 000013.           WXX=WXX+X(I)*X(I)*W(I)
20 000014.           WXY=WXY+X(I)*Y(I)*W(I)
21 000015.     777   WYY=WYY+Y(I)*Y(I)*W(I)
22 000016.           DEN=WW*WXX-WX*WX
23 000017.           SLOPE=(WW*WXY-WX*WY)/DEN
24 000018.           YINT=(WXX*WY-WX*WXY)/DEN
25 000019.           VSUM=0.0
26 000020.           DO 888 I=1,N
27 000021.     888   VSUM=VSUM+(Y(I)-YINT-SLOPE*X(I))**2
28 000022.           AN=N-2
29 000023.           SS=VSUM/AN
30 000024.           SDFIT=SQRT(SS)
31 000025.           SDS=SQRT(SS*WW/DEN)
32 000026.           SDINT=SQRT(SS/WW*(1.0+((WX*WX)/DEN)))
33 000027.           TERM=SQRT(DEN*(WW*WYY-WY*WY))
34 000028.           CORR=(WW*WXY-WX*WY)/TERM
35 000029.           RETURN
36 000030.           END
37
38
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57
```
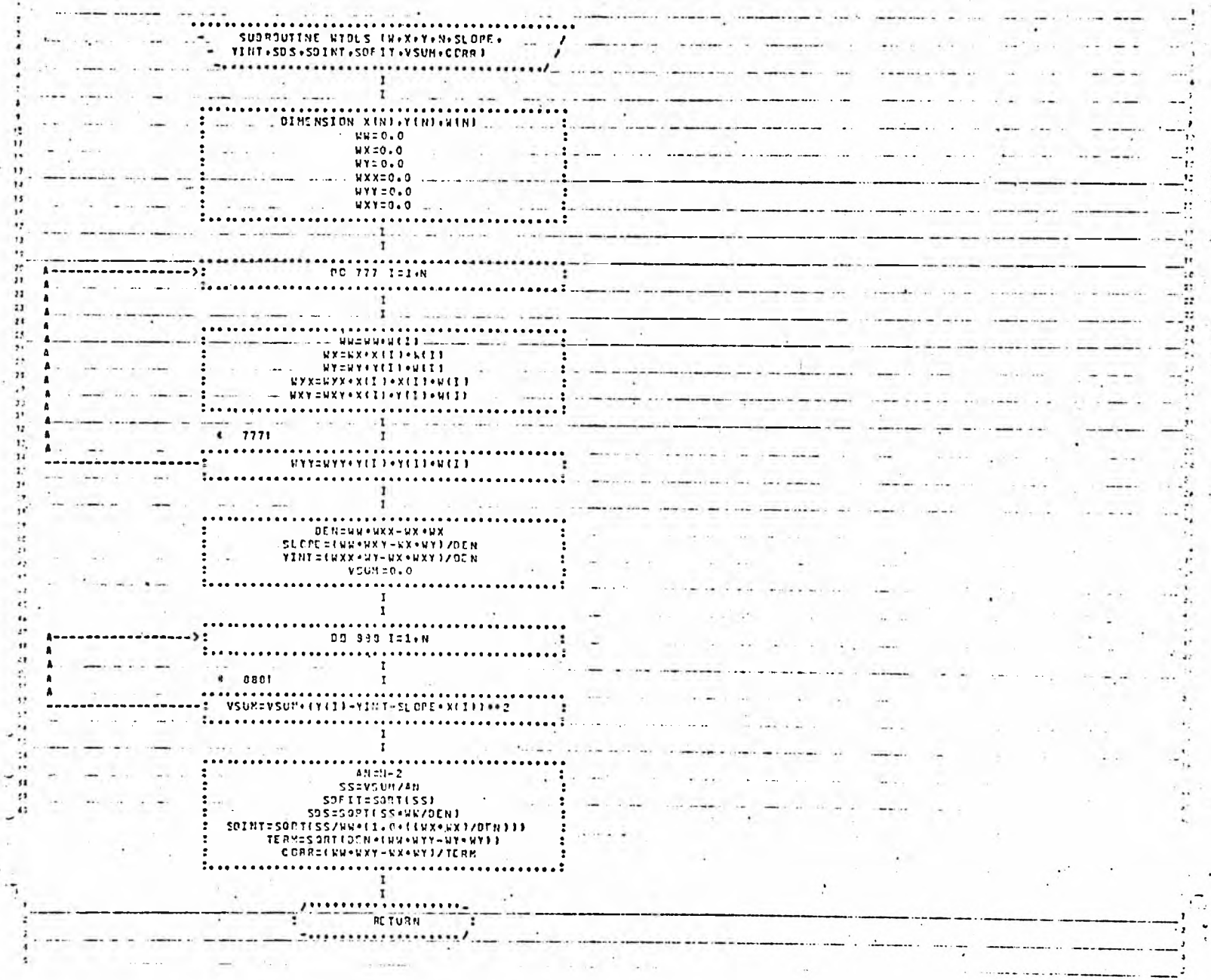
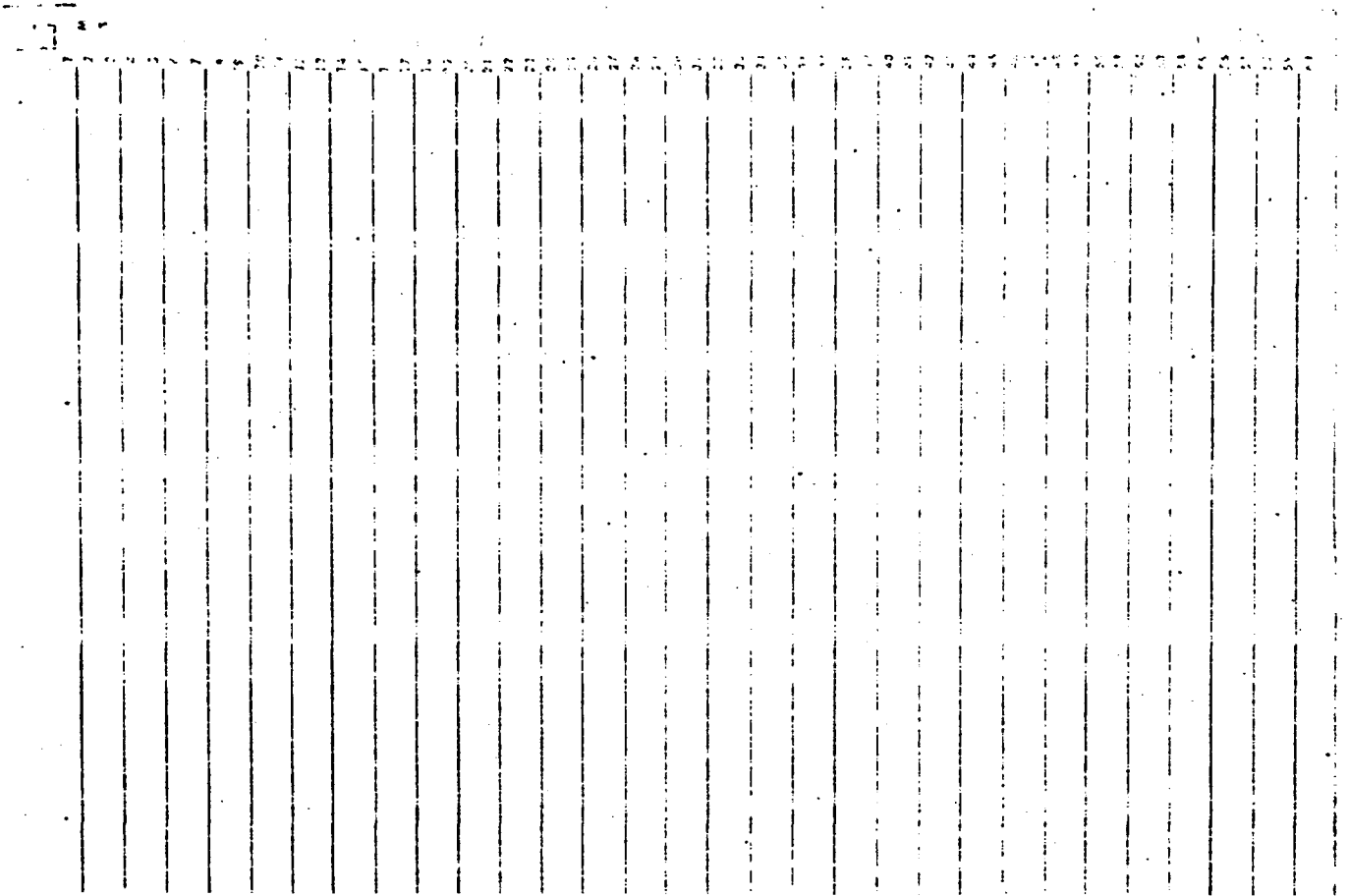
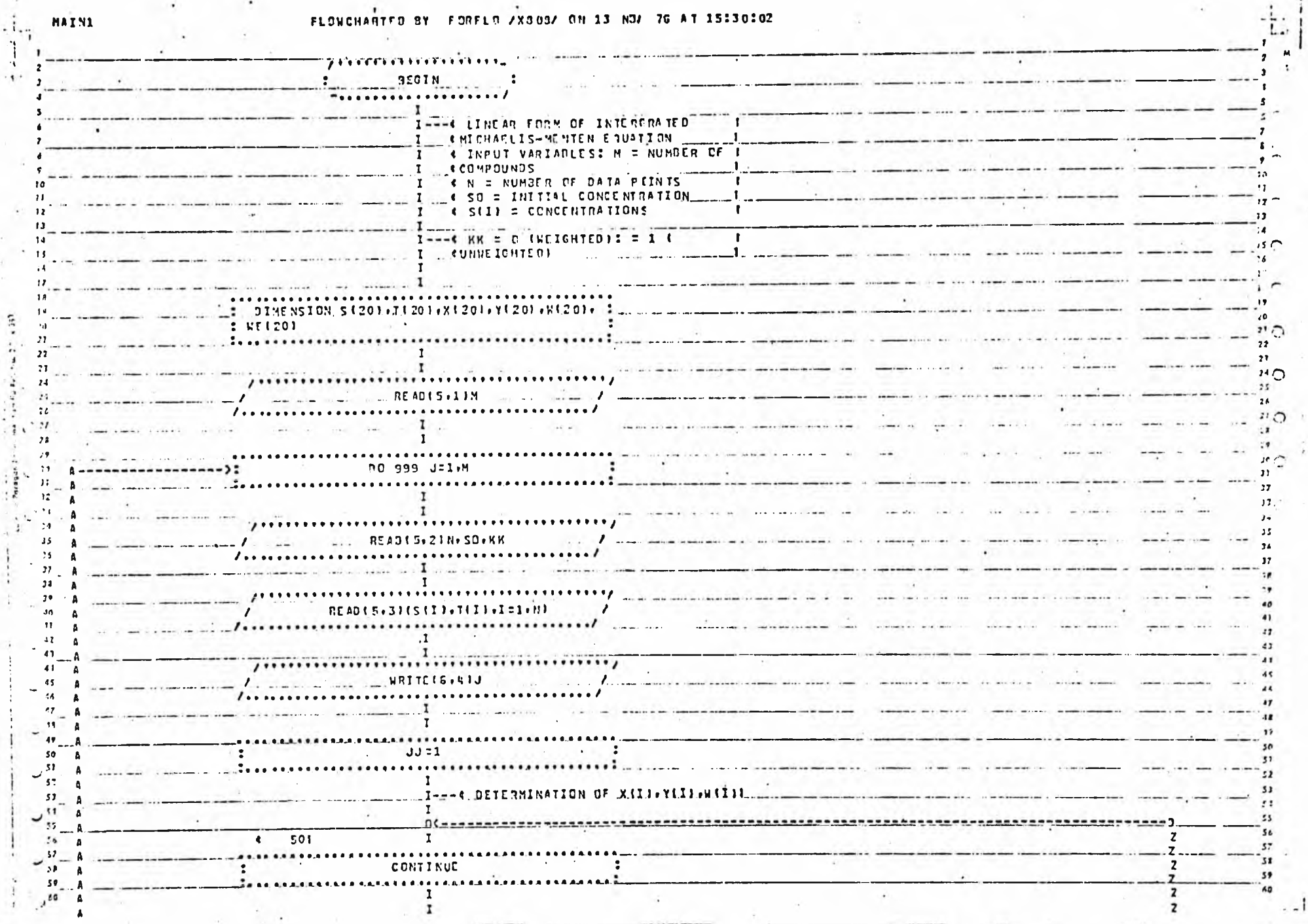


SUBROUTINE WTDLS (W,X,Y,N,SLOPE,
YINT,SDS,SDINT,SDFIT,VSUM,CORR)
DIMENSION X(N),Y(N),W(N)
WW=0.0
WX=0.0
WY=0.0
WXX=0.0
WYY=0.0
WXY=0.0
DO 777 I=1,N
WW=WW+W(I)
WX=WX+X(I)*W(I)
WY=WY+Y(I)*W(I)
WXX=WXX+X(I)*X(I)*W(I)
WXY=WXY+X(I)*Y(I)*W(I)
777
WYY=WYY+Y(I)*Y(I)*W(I)
DEN=WW*WXX-WX*WX
SLOPE=(WW*WXY-WX*WY)/DEN
YINT=(WXX*WY-WX*WXY)/DEN
VSUM=0.0
DO 880 I=1,N
VSUM=VSUM+(Y(I)-YINT-SLOPE*X(I))**2
880
AN=N-2
SS=VSUM/AN
SDFIT=SQRT(SS)
SDS=SQRT(SS*WW/DEN)
SDINT=SQRT(SS/WW*(1.0+((WX*WX)/DEN)))
TERM=SQRT(DEN*(WW*WYY-WY*WY))
CORR=(WW*WXY-WX*WY)/TERM
RETURN

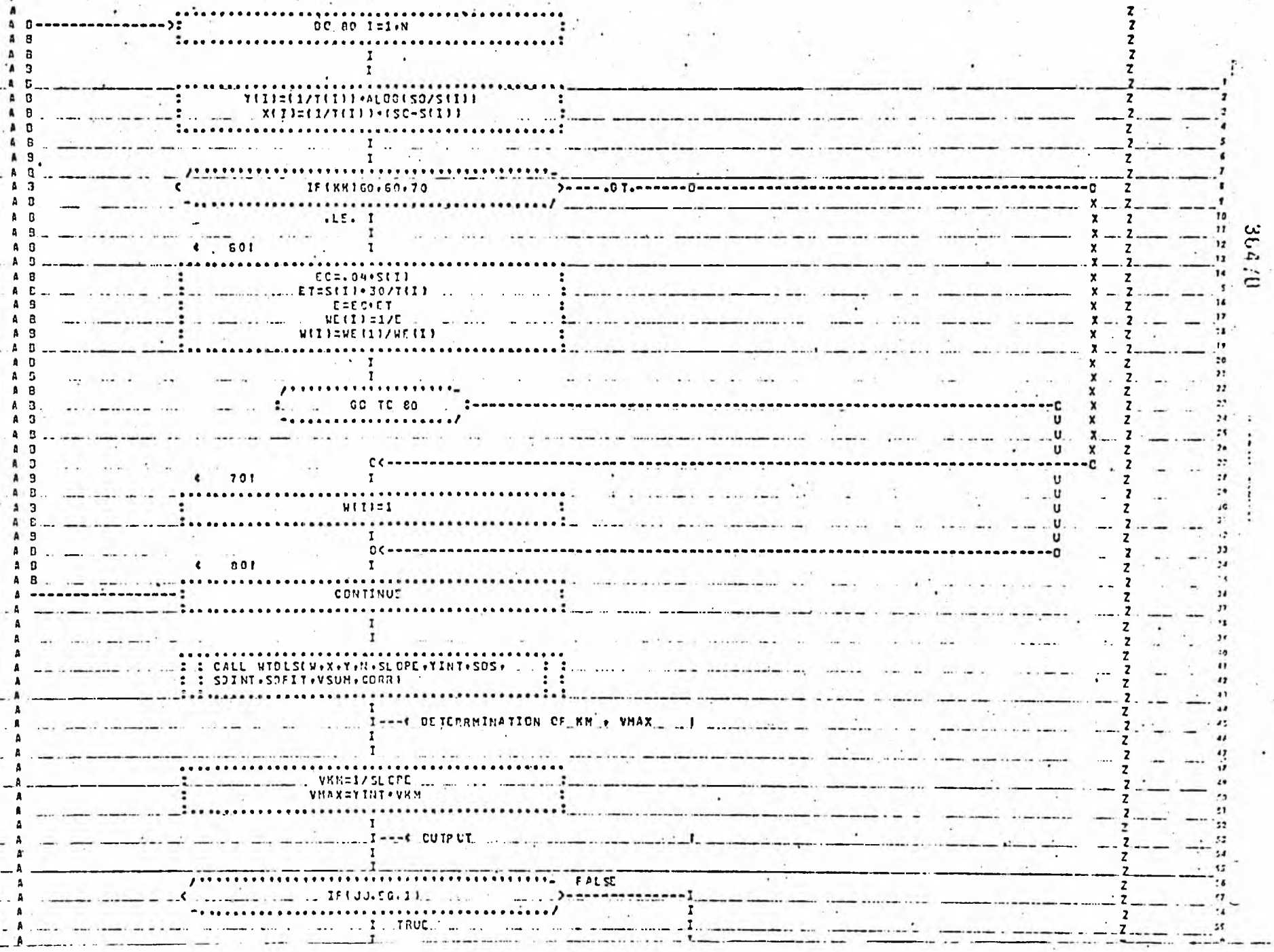
```
 GE8*UPLI.FORFLO,S JA.MAIN1
 FLOWCHARTED BY  FCRFLC /X808/ ON 13 NOV 70 AT 15:29:36

 000001.      C      LINEAR FORM OF INTEGRATED MICHAELIS-MENTEN EQUATION
 000002.      C      INPUT VARIABLES: M = NUMBER OF COMPOUNDS
 000003.      C                       N = NUMBER OF DATA POINTS
 000004.      C                      SO = INITIAL CONCENTRATION
 000005.      C                    S(I) = CONCENTRATIONS
 000006.      C                    T(I) = TIMES
 000007.      C                      KK = 0 (WEIGHTED); = 1 (UNWEIGHTED)
 000009.            DIMENSION S(20),T(20),X(20),Y(20),W(20),WE(20)
 000009.            READ(5,1)M
 000010.            DO 999 J=1,M
 000011.            READ(5,2)N,SO,KK
 000012.            READ(5,3)(S(I),T(I),I=1,N)
 000013.            WRITE(6,4)J
 000014.            JJ=1
 000015.      C     DETERMINATION OF X(I),Y(I),W(I)
 000016.         50 CONTINUE
 000017.            DO 90 I=1,N
 000018.            Y(I)=(1/T(I))*ALOG(SO/S(I))
 000019.            X(I)=(1/T(I))*(SO-S(I))
 000020.            IF(KK)60,60,70
 000021.         60 EC=.04*S(I)
 000022.            ET=S(I)*30/T(I)
 000023.            E=EC+ET
 000024.            WE(I)=1/E
 000025.            W(I)=WE(I)/WE(I)
 000026.            GO TO 80
 000027.         70 W(I)=1
 000028.         80 CONTINUE
 000029.            CALL WTDLS(W,X,Y,N,SLOPE,YINT,SDS,SDINT,SDFIT,VSUM,CORR)
 000030.      C     DETERMINATION OF KM & VMAX
 000031.            VKM=1/SLOPE
 000032.            VMAX=YINT*VKM
 000033.      C     OUTPUT
 000034.            IF(JJ.EQ.1)WRITE(6,5)
 000035.            IF(JJ.EQ.2)WRITE(6,6)
 000036.            IF(JJ.EQ.3)WRITE(6,7)
 000037.            WRITE(6,8)SLOPE,SDS
 000038.            WRITE(6,9)YINT,SDINT
 000039.            WRITE(6,10)SDFIT,CORR
 000040.            WRITE(6,11)VKM,VMAX
 000041.      C     DETERMINATION OF UPPER & LOWER LIMITS
 000042.            JJ=JJ+1
 000043.            DO 100 I=1,N
 000044.            IF(JJ.EQ.2)S(I)=S(I)*.96
 000045.            IF(JJ.EQ.3)S(I)=S(I)/.96*1.04
 000046.        100 CONTINUE
 000047.            IF(JJ.LT.4)GO TO 50
 000048.          1 FORMAT(I)
 000049.          2 FORMAT(I3,F7.4,I3)
 000050.          3 FORMAT(8.6,F6.1)
 000051.          4 FORMAT(10X,'COMPOUND NUMBER :  ',I2,///)
```

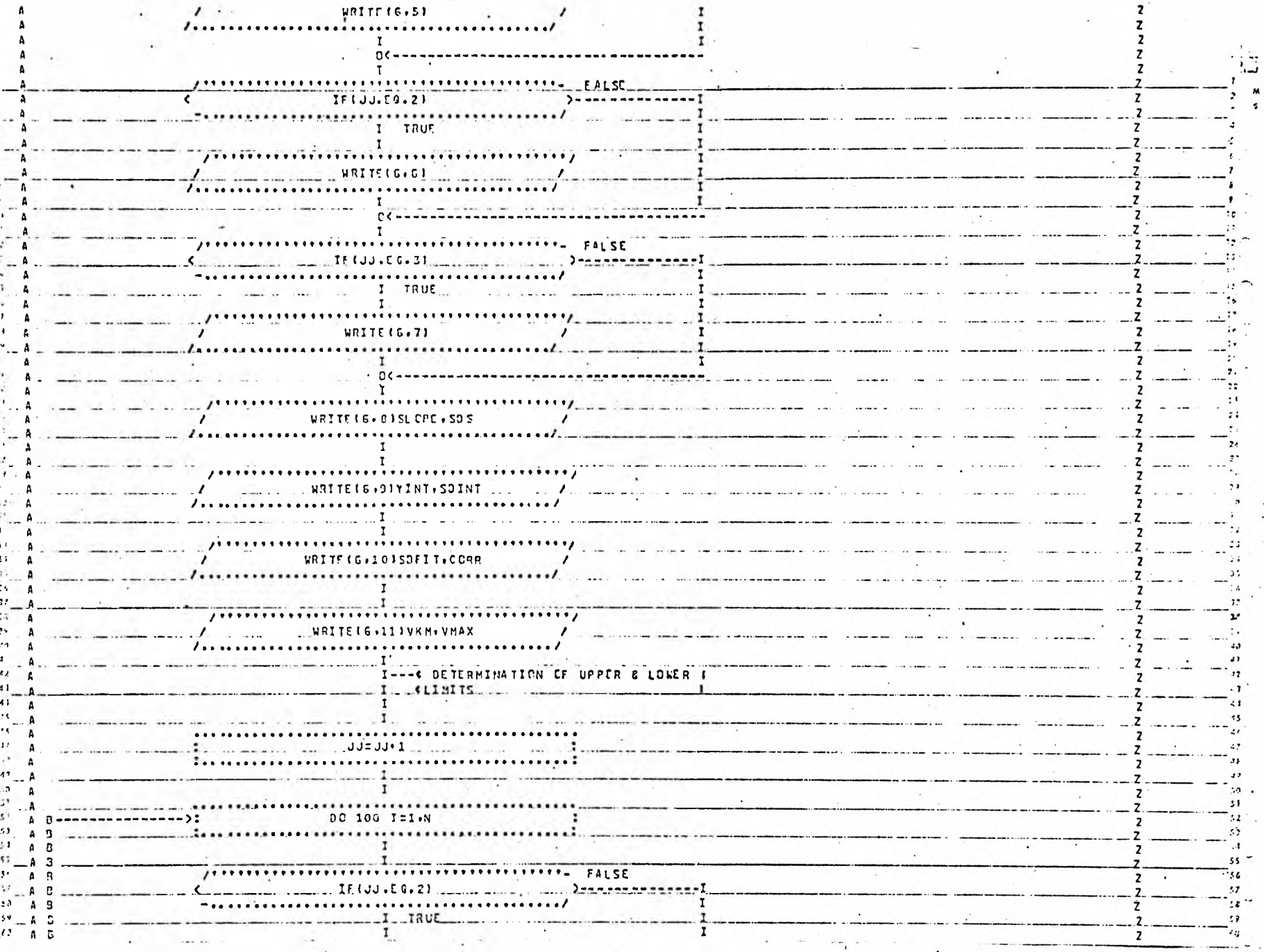


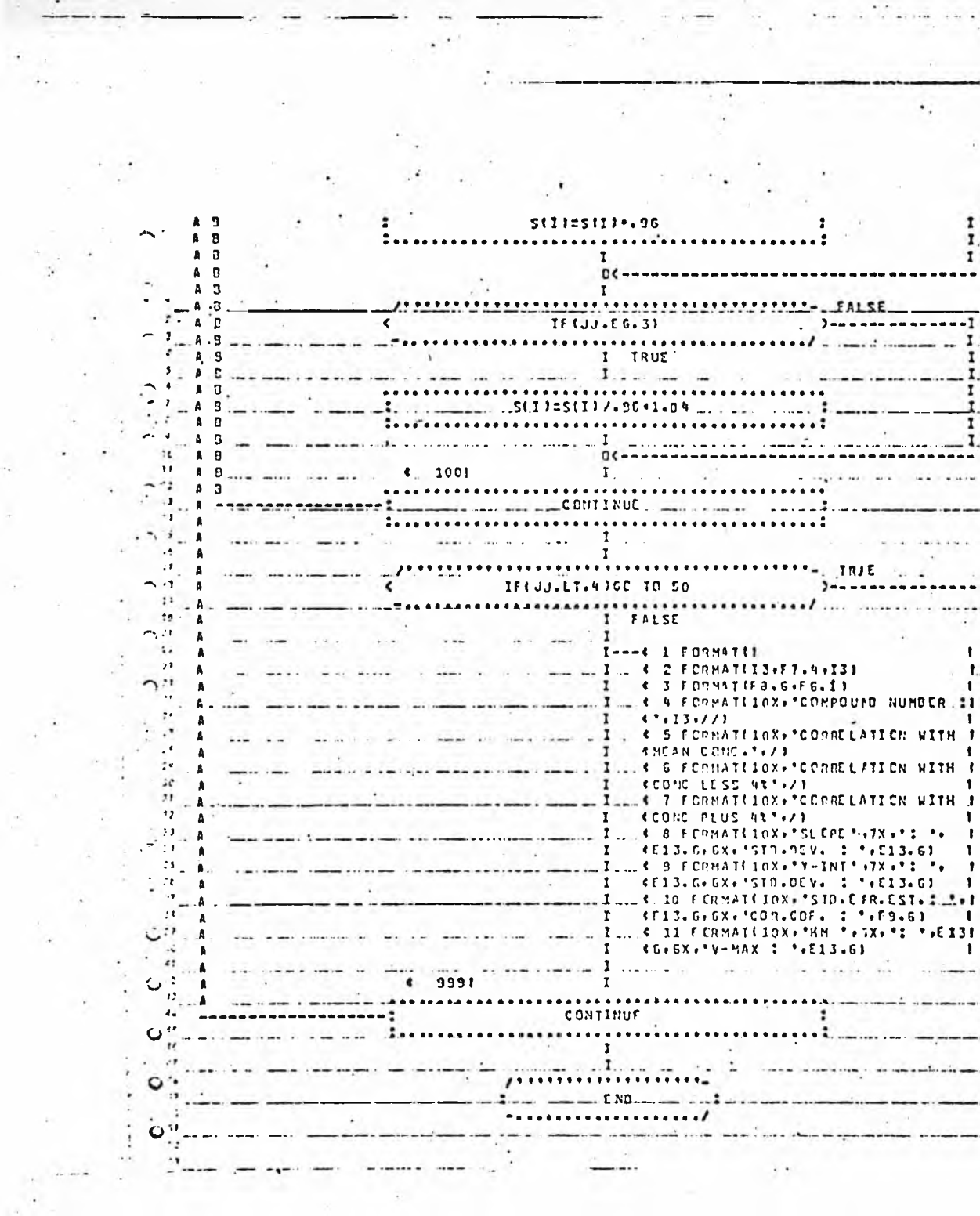
```
000052.          5 FORMAT(10X,'CORRELATION WITH MEAN CONC.',/)
000053.          6 FORMAT(10X,'CORRELATION WITH CONC LESS 4%',/)
000054.          7 FORMAT(10X,'CORRELATION WITH CONC PLUS 4%',/)
000055.          8 FORMAT(10X,'SLOPE ',7X,': ',E13.6,6X,'STD.DEV. :
000056.          9 FORMAT(10X,'Y-INT ',7X,': ',E13.6,6X,'STD.DEV. :
000057.         10 FORMAT(10X,'STD.ERR.EST.: ',E13.6,6X,'COR.COE. :
000058.         11 FORMAT(10X,'KM    ',7X,': ',E13.6,6X,'V-MAX    :
000059.        999 CONTINUE
000060.            END
```

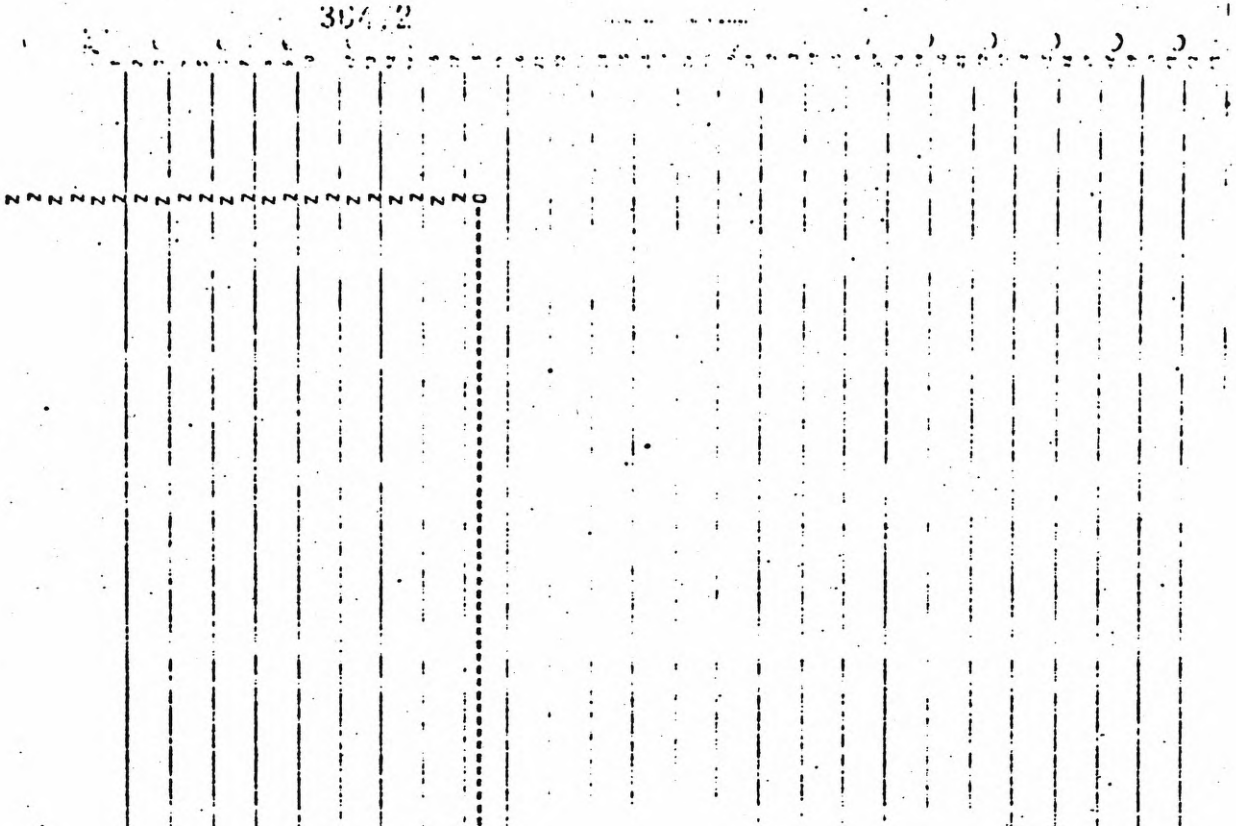
```
',E13.6)
',E13.6)
',F9.6)
',E13.6)
```


MAIN1
FLOWCHARTED BY FORFLO /X309/ ON 13 NOV 76 AT 15:30:02
BEGIN
LINEAR FORM OF INTEGRATED MICHAELIS-MENTEN EQUATION
INPUT VARIABLES: M = NUMBER OF COMPOUNDS
N = NUMBER OF DATA POINTS
SO = INITIAL CONCENTRATION
S(I) = CONCENTRATIONS
KK = 0 (WEIGHTED): = 1 (UNWEIGHTED)
DIMENSION S(20),T(20),X(20),Y(20),W(20),WE(20)
READ(5,1)M
DO 999 J=1,M
READ(5,2)N,SO,KK
READ(5,3)(S(I),T(I),I=1,N)
WRITE(6,4)J
JJ=1
DETERMINATION OF X(I),Y(I),W(I)
501
CONTINUE

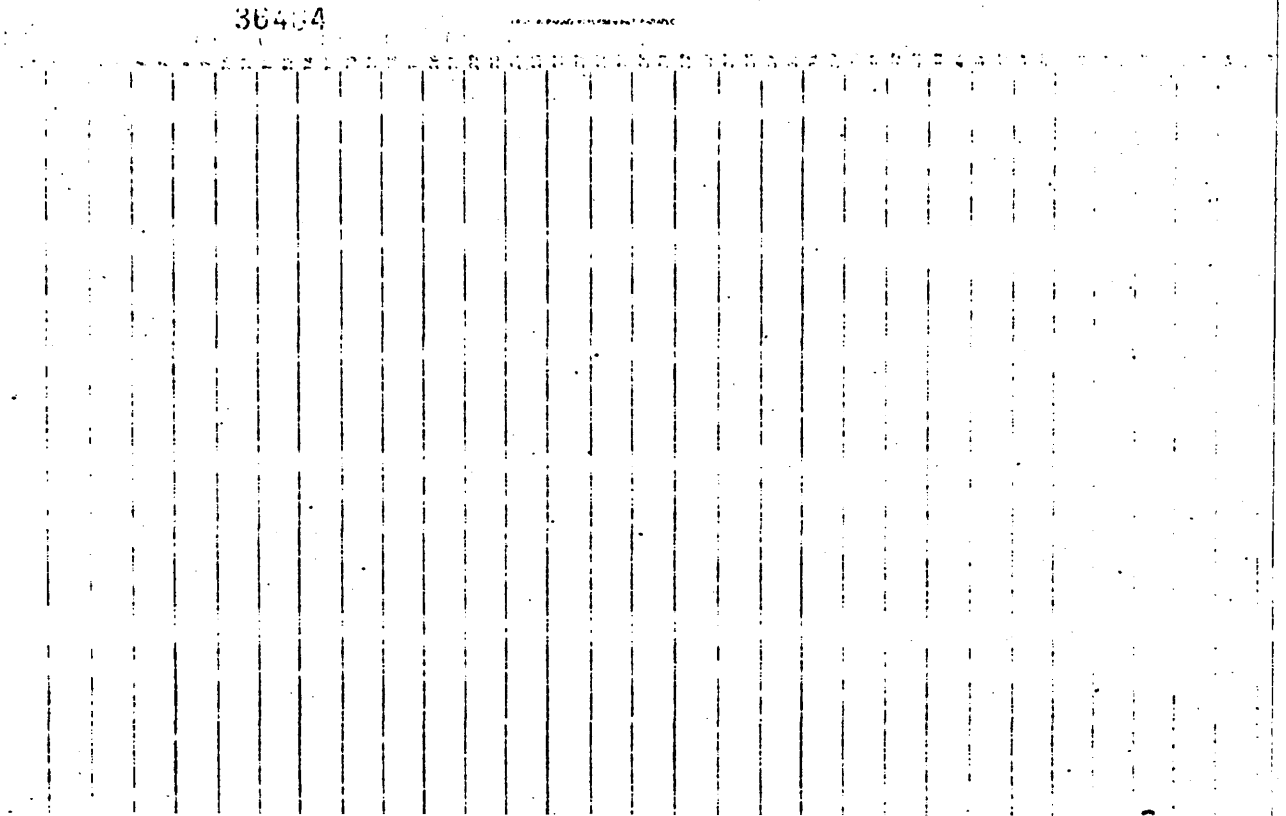

DO 80 I=1,N
Y(I)=(1/T(I))*ALOG(SO/S(I))
X(I)=(1/T(I))*(SC-S(I))
IF(KM)60,60,70
.LE.
60
EC=.04*S(I)
ET=S(I)*30/T(I)
E=EC+ET
WE(I)=1/E
W(I)=WE(I)/WE(I)
GO TO 80
70
W(I)=1
80
CONTINUE
CALL WTDLS(W,X,Y,N,SLOPE,YINT,SDS,
SDINT,SDFIT,VSUM,CORR)
DETERMINATION OF KM , VMAX
VKM=1/SLOPE
VMAX=YINT*VKM
OUTPUT
IF(JJ.EQ.1)
TRUE
FALSE

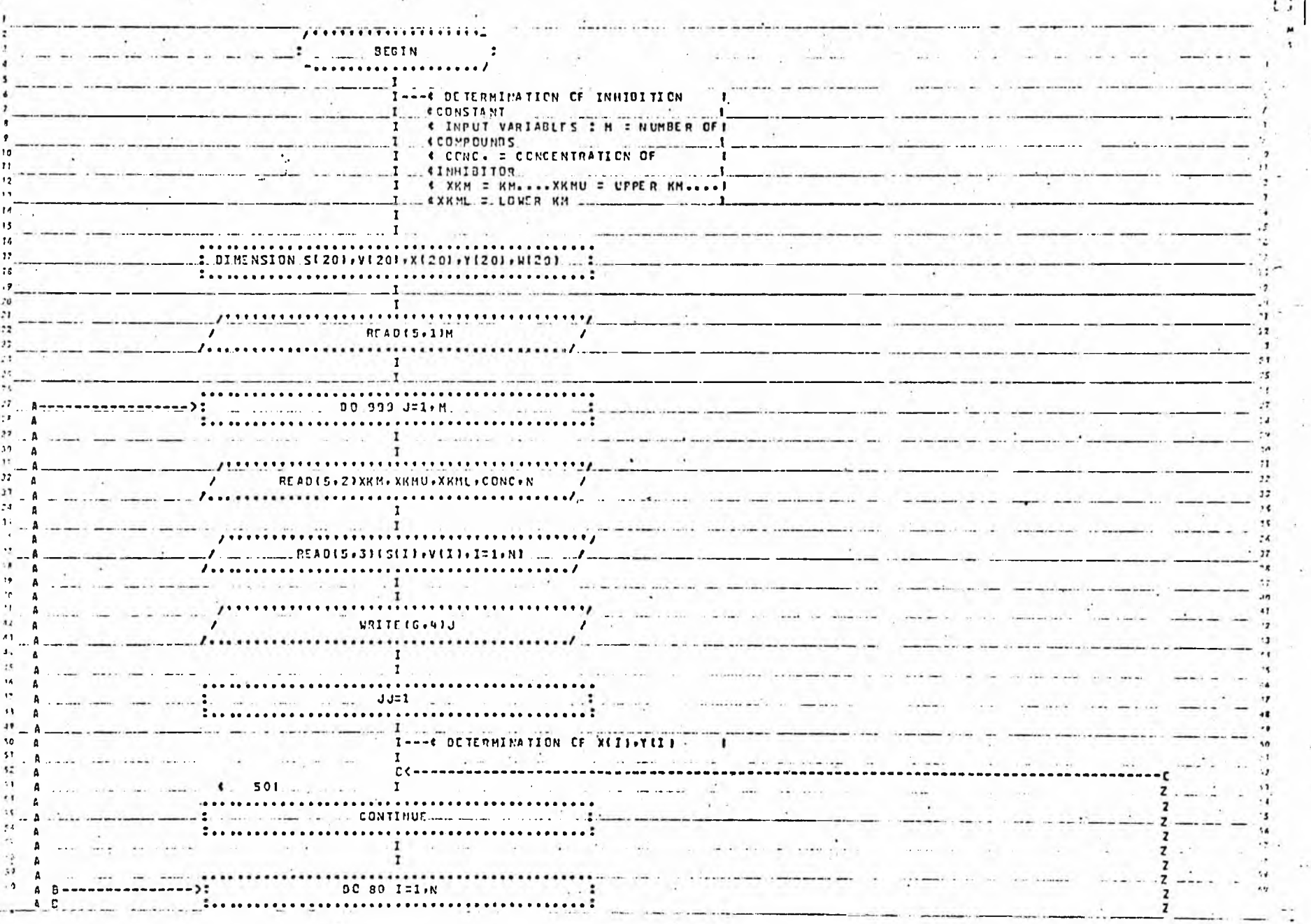

WRITE(6,5)
IF(JJ.EQ.2)
FALSE
TRUE
WRITE(6,6)
IF(JJ.EQ.3)
FALSE
TRUE
WRITE(6,7)
WRITE(6,8)SLOPE,SDS
WRITE(6,9)YINT,SDINT
WRITE(6,10)SDFIT,CORR
WRITE(6,11)VKM,VMAX
DETERMINATION OF UPPER & LOWER
LIMITS
JJ=JJ+1
DO 100 I=1,N
IF(JJ.EQ.2)
FALSE
TRUE

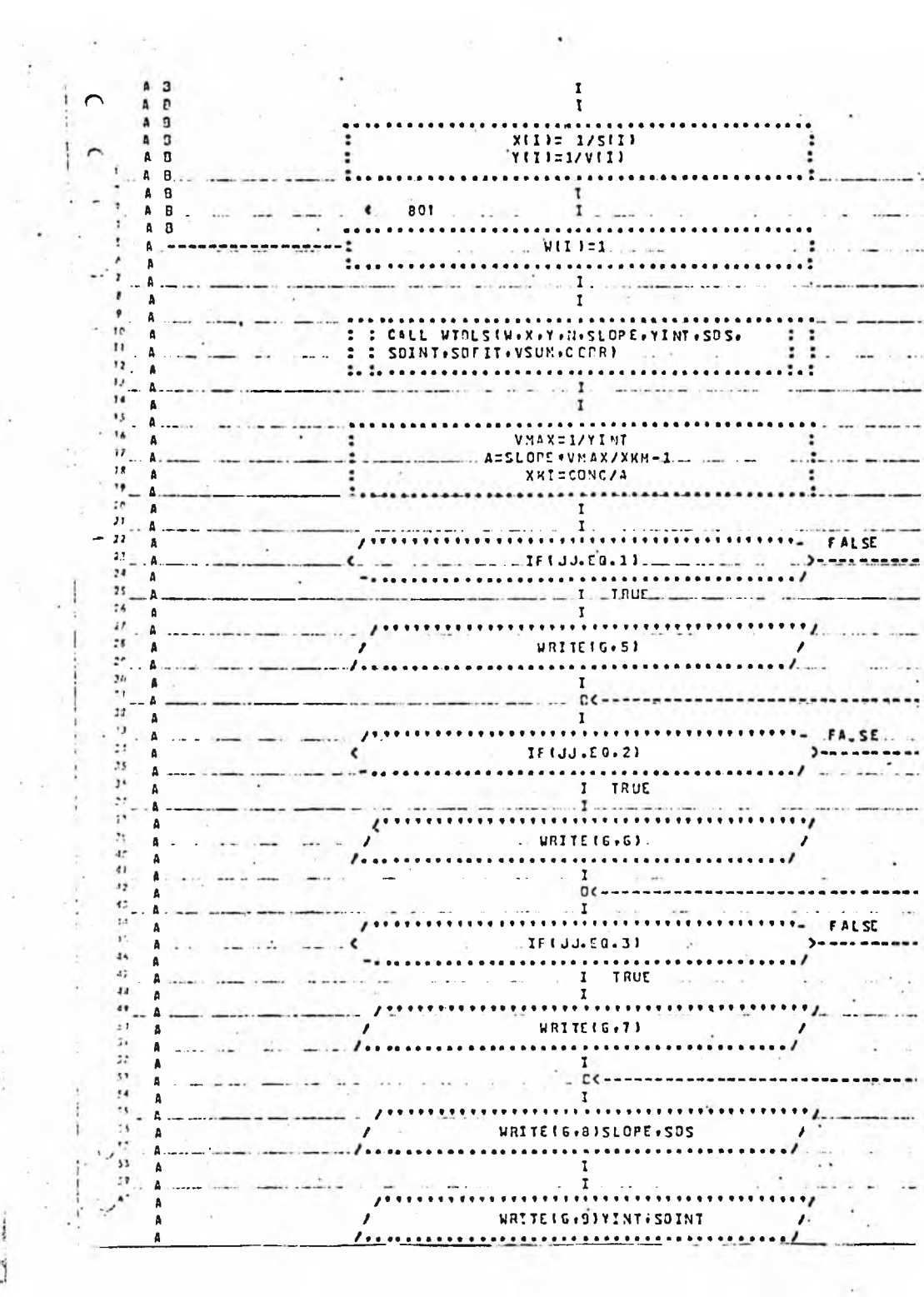

S(I)=S(I)*.96
IF(JJ.EQ.3)
TRUE
FALSE
S(I)=S(I)/.90*1.04
100
CONTINUE
IF(JJ.LT.4)GO TO 50
TRUE
FALSE
1 FORMAT(1
2 FORMAT(I3,F7.4,I3)
3 FORMAT(F8.6,F6.1)
4 FORMAT(10X,'COMPOUND NUMBER
(',I3,//)
5 FORMAT(10X,'CORRELATION WITH
(MEAN CONC.',/)
6 FORMAT(10X,'CORRELATION WITH
(CONC LESS %',/)
7 FORMAT(10X,'CORRELATION WITH
(CONC PLUS %',/)
8 FORMAT(10X,'SLOPE',7X,': ',
(E13.6,6X,'STD.DEV. : ',E13.6)
9 FORMAT(10X,'Y-INT',7X,': ',
(E13.6,6X,'STD.DEV. : ',E13.6)
10 FORMAT(10X,'STD.ERR.EST. : ',
(F13.6,6X,'COR.COF. : ',F9.6)
11 FORMAT(10X,'RM ',7X,': ',E13
(6,6X,'Y-MAX : ',E13.6)
999
CONTINUE
END

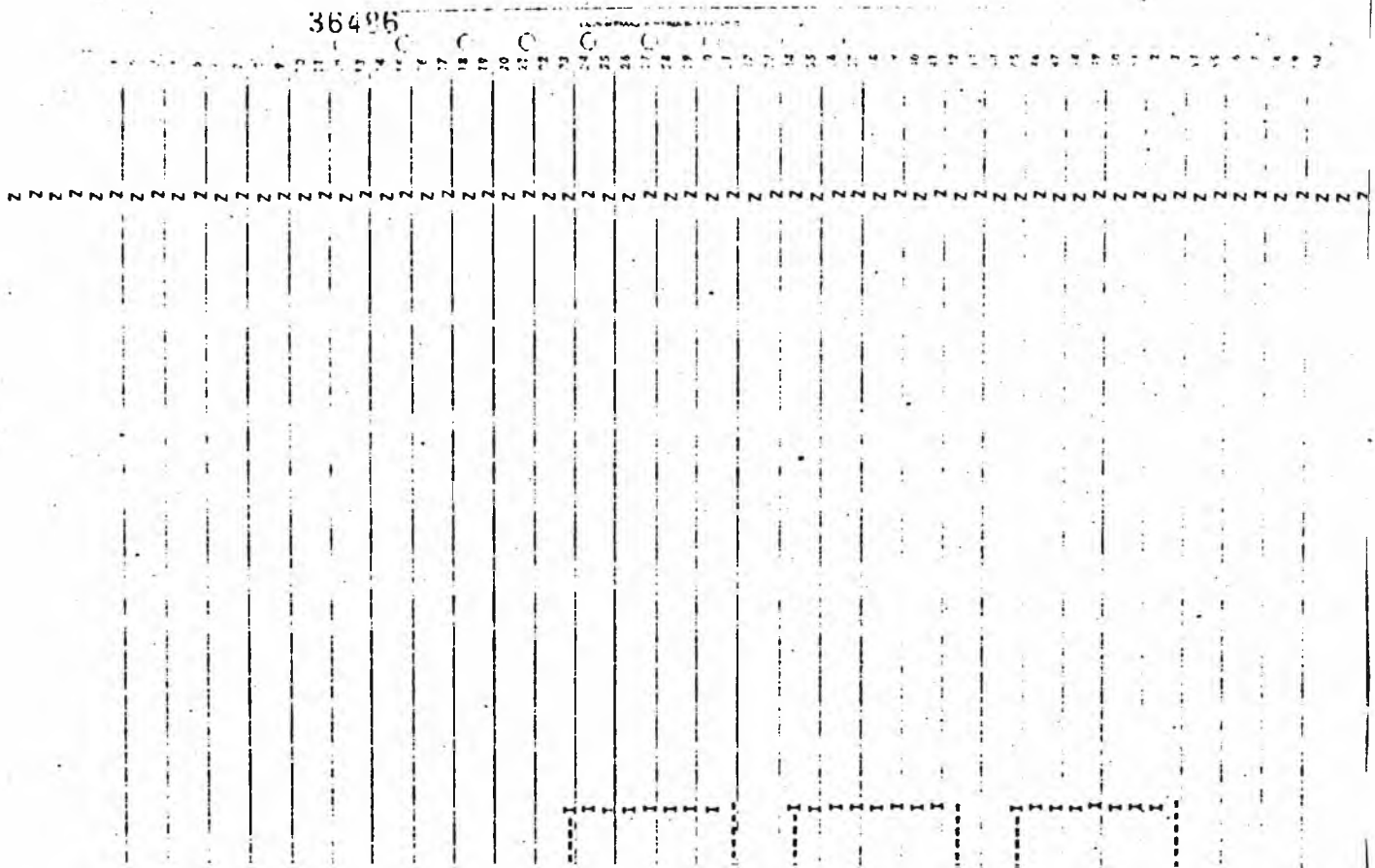

304.2

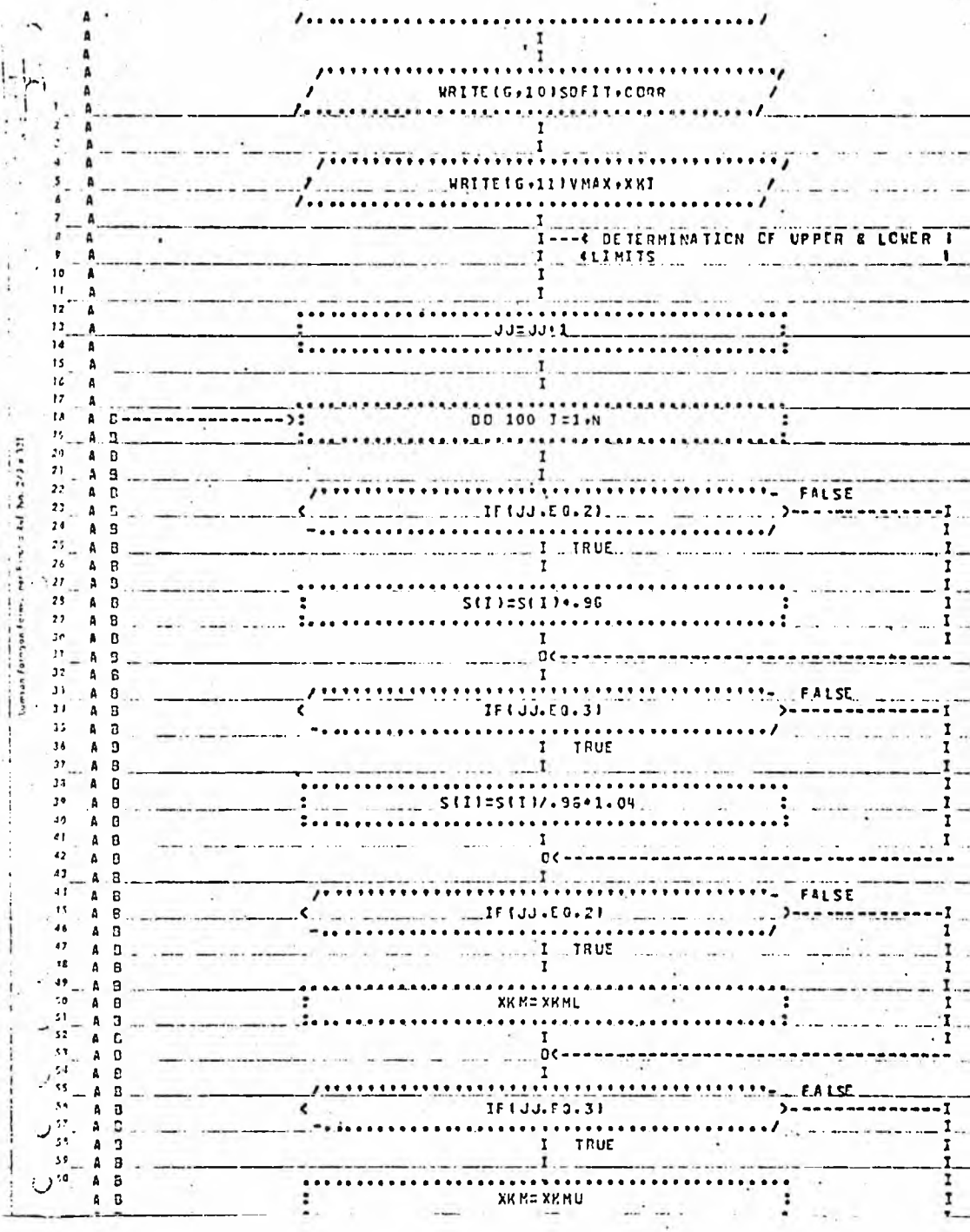
```
@ER*UPLI.FORFLO.S JA.MAIN2
FLOWCHARTED BY  FORFLC /X808/ ON 13 NOV 76 AT 15:31:30


000001.      C     DETERMINATION OF INHIBITION CONSTANT
000002.      C     INPUT VARIABLES : M = NUMBER OF COMPOUNDS
000003.      C                       CONC. = CONCENTRATION OF INHIBITOR
000004.      C                         XKM = KM....XKMU = UPPER KM....XKML = LOWER KM
000005.            DIMENSION S(20),V(20),X(20),Y(20),W(20)
000006.            READ(5,1)M
000007.            DO 999 J=1,M
000008.            READ(5,2)XKM,XKMU,XKML,CONC,N
000009.            READ(5,3)(S(I),V(I),I=1,N)
000010.            WRITE(6,4)J
000011.            JJ=1
000012.      C     DETERMINATION OF X(I),Y(I)
000013.         50 CONTINUE
000014.            DO 80 I=1,N
000015.            X(I)= 1/S(I)
000016.            Y(I)=1/V(I)
000017.         80 W(I)=1
000018.            CALL WTDLS(W,X,Y,N,SLOPE,YINT,SDS,SDINT,SDFIT,VSUM,CORR)
000019.            VMAX=1/YINT
000020.            A=SLOPE*VMAX/XKM-1
000021.            XKI=CONC/A
000022.            IF(JJ.EQ.1)WRITE(6,5)
000023.            IF(JJ.EQ.2)WRITE(6,6)
000024.            IF(JJ.EQ.3)WRITE(6,7)
000025.            WRITE(6,8)SLOPE,SDS
000026.            WRITE(6,9)YINT,SDINT
000027.            WRITE(6,10)SDFIT,CORR
000028.            WRITE(6,11)VMAX,XKI
000029.      C     DETERMINATION OF UPPER & LOWER LIMITS
000030.            JJ=JJ+1
000031.            DO 100 I=1,N
000032.            IF(JJ.EQ.2)S(I)=S(I)*.98
000033.            IF(JJ.EQ.3)S(I)=S(I)/.98*1.04
000034.            IF(JJ.EQ.2)XKM=XKML
000035.            IF(JJ.EQ.3)XKM=XKMU
000036.        100 CONTINUE
000037.            IF(JJ.LT.4)GO TO 50
000038.          1 FORMAT()
000039.          2 FORMAT(F7.5,I3)
000040.          3 FORMAT(F8.6,F8.3)
000041.          4 FORMAT(10X,'COMPOUND NUMBER :  ',I3,//)
000042.          5 FORMAT(10X,'CORRELATION USING MEAN CONC. ',/)
000043.          6 FORMAT(10X,'CORRELATION USING MEAN CONC.- 4%',/)
000044.          7 FORMAT(10X,'CORRELATION USING MEAN CONC.+ 4%',/)
000045.          8 FORMAT(10X,'SLOPE          :',2X,E13.6,6X,'STD.DEV ',2X,E13.6)
000046.          9 FORMAT(10X,'YINTERCEPT     :',2X,E13.6,6X,'STD.DEV ',2X,E13.6)
000047.         10 FORMAT(10X,'STD.ERR.EST.:',2X,E13.6,6X,'CORR.COEF.',2X,F9.6,/)
000048.         11 FORMAT(10X,'V-MAX         :',2X,E13.6,6X,'KI      :',2X,E13.6,///
000049.        999 CONTINUE
000050.            END
```

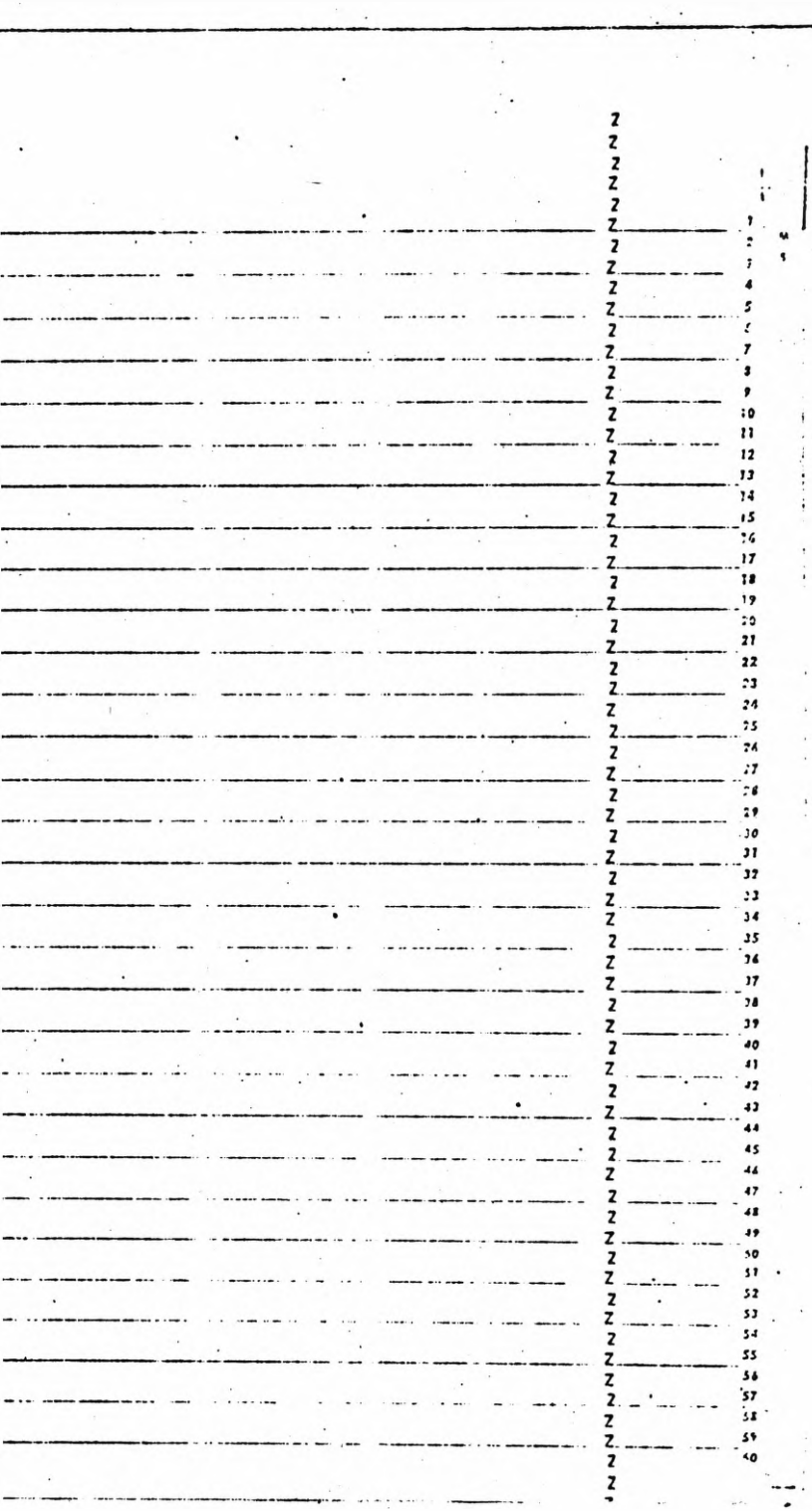

36434

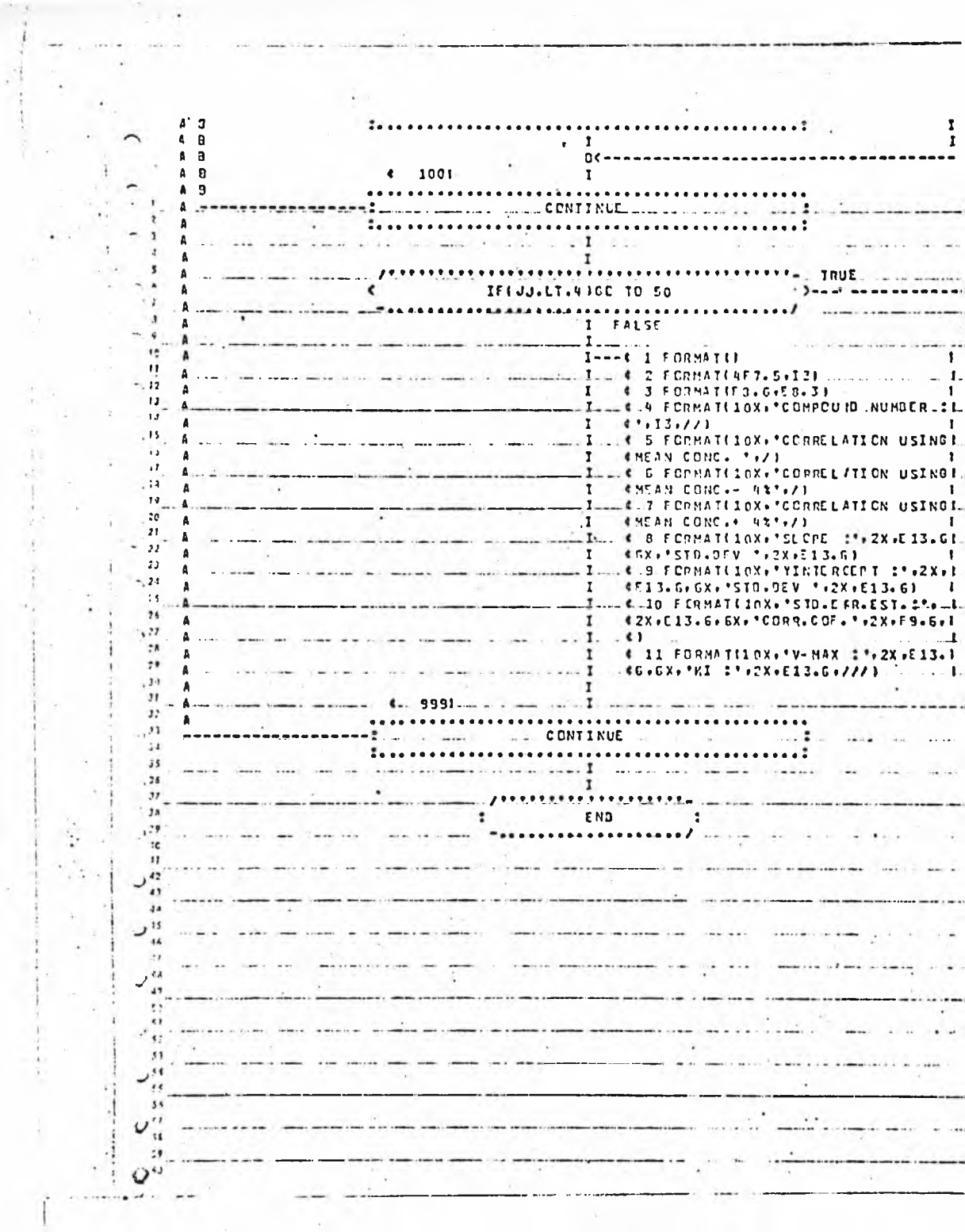

BEGIN
C DETERMINATION OF INHIBITION
C CONSTANT
C INPUT VARIABLES : M = NUMBER OF
C COMPOUNDS
C CONC. = CONCENTRATION OF
C INHIBITOR
C XKM = KM....XKMU = UPPER KM....
C XKML = LOWER KM
DIMENSION S(20),V(20),X(20),Y(20),W(20)
READ(5,1)M
DO 999 J=1,M
READ(5,2)XKM,XKMU,XKML,CONC,N
READ(5,3)(S(I),V(I),I=1,N)
WRITE(6,4)J
JJ=1
C DETERMINATION OF X(I),Y(I)
501
CONTINUE
DO 80 I=1,N

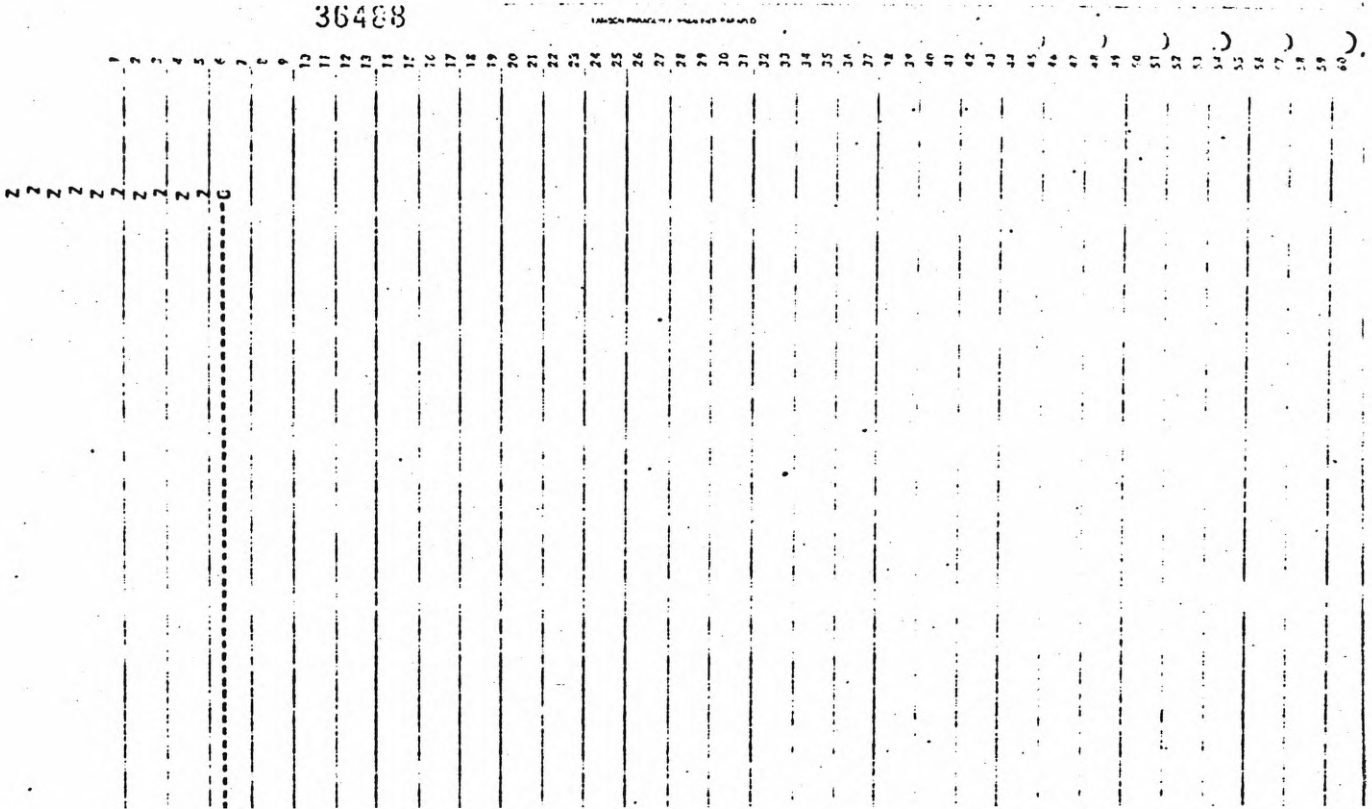

X(I)= 1/S(I)
Y(I)=1/V(I)
801
W(I)=1
CALL WTOLS(W,X,Y,N,SLOPE,YINT,SDS,
SDINT,SDFIT,VSUM,CORR)
YMAX=1/YINT
A=SLOPE*YMAX/XKM-1
XKI=CONC/A
IF(JJ.EQ.1)
FALSE
TRUE
WRITE(6,5)
IF(JJ.EQ.2)
FALSE
TRUE
WRITE(6,6)
IF(JJ.EQ.3)
FALSE
TRUE
WRITE(6,7)
WRITE(6,8)SLOPE,SDS
WRITE(6,9)YINT,SDINT


36496


WRITE(6,10)SOFIT,CORR
WRITE(6,11)VMAX,XKI
DETERMINATION OF UPPER & LOWER LIMITS
JJ=JJ+1
DO 100 I=1,N
IF(JJ.EQ.2)
FALSE
TRUE
S(I)=S(I)*.96
IF(JJ.EQ.3)
FALSE
TRUE
S(I)=S(I)/.96*1.04
IF(JJ.EQ.2)
FALSE
TRUE
XKM=XKML
IF(JJ.EQ.3)
FALSE
TRUE
XKM=XKMU

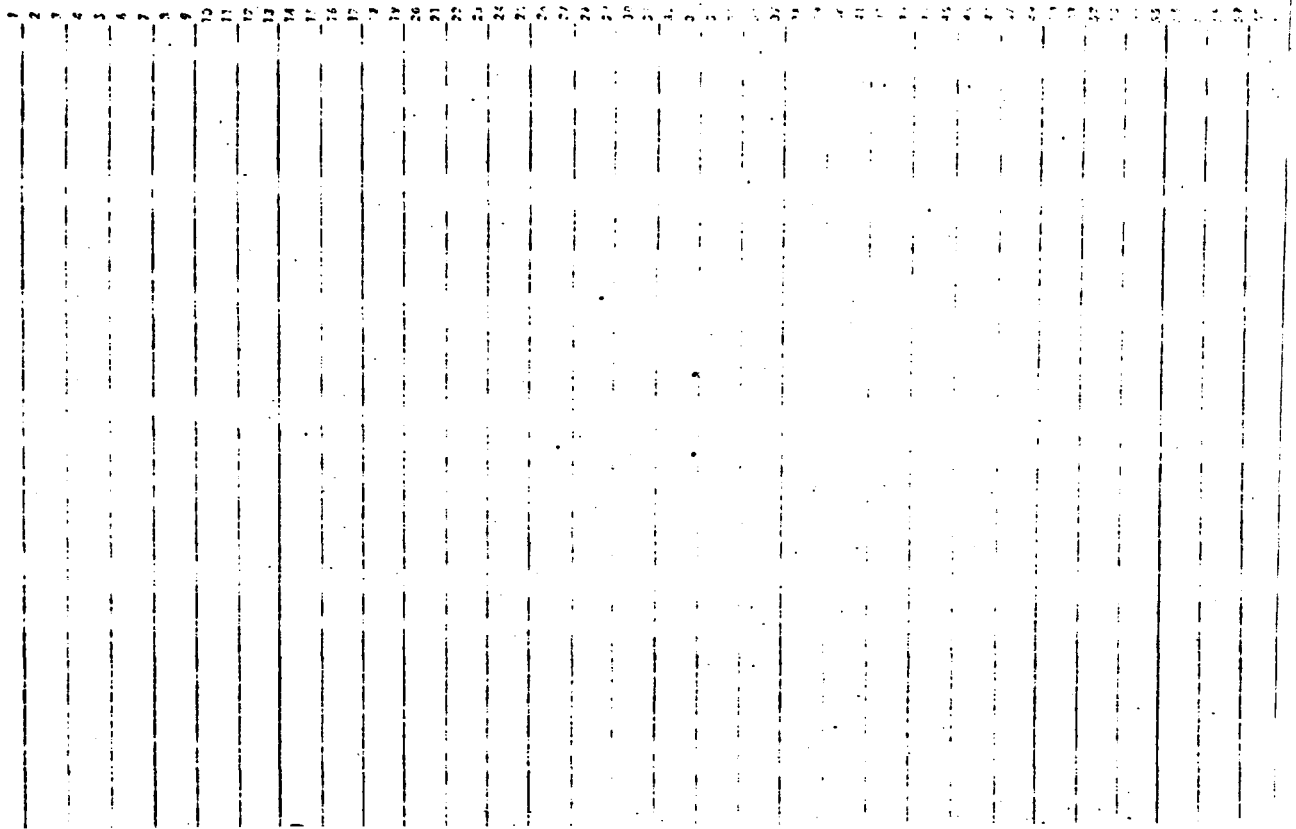



1001
CONTINUE
IF(JJ.LT.4)GO TO 50
TRUE
FALSE
9999
CONTINUE
END

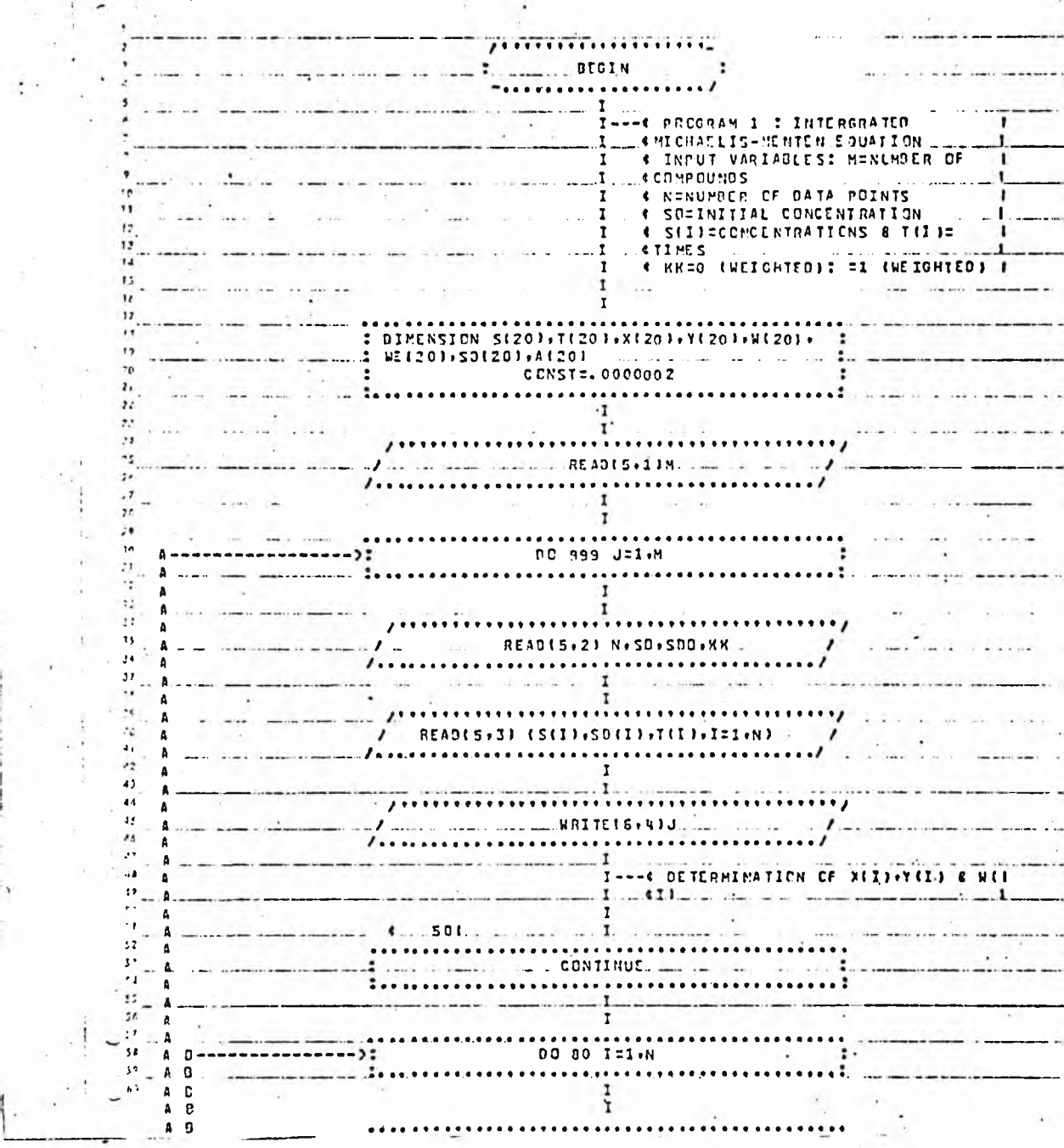

36488

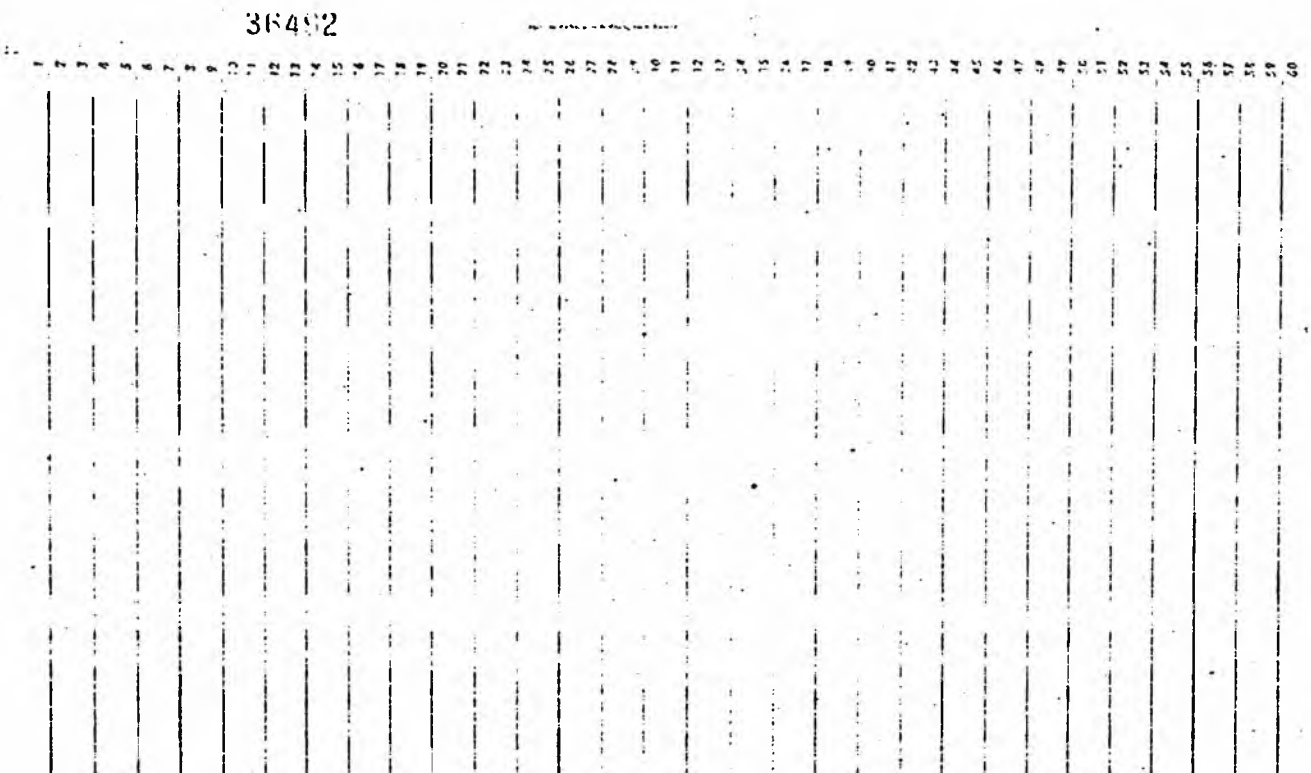
```
GEB*UPLI.FORFLO,S JA.MAIN$
FLOWCHARTED BY FORFLC /X808/ ON 13 NOV 70 AT 15:32:10

000001.      C      PROGRAM 1 : INTEGRATED MICHAELIS-MENTEN EQUATION
000002.      C      INPUT VARIABLES: M=NUMBER OF COMPOUNDS
000003.      C                       N=NUMBER OF DATA POINTS
000004.      C                       SO=INITIAL CONCENTRATION
000005.      C                       S(I)=CONCENTRATIONS & T(I)=TIMES
000006.      C                       KK=0 (WEIGHTED); =1 (WEIGHTED)
000007.            DIMENSION S(20),T(20),X(20),Y(20),W(20),WE(20),SO(20),A(20)
000008.            CONST=.0000002
000009.            READ(5,1)M
000010.            DO 999 J=1,M
000011.            READ(5,2) N,SO,SOO,KK
000012.            READ(5,3) (S(I),SO(I),T(I),I=1,N)
000013.            WRITE(6,4)J
000014.      C     DETERMINATION OF X(I),Y(I) & W(I)
000015.         50 CONTINUE
000016.            DO 30 I=1,N
000017.            X(I)=ALOG(SO/S(I))-ALOG(SOO/SO(I))
000018.            Y(I)=(S(I)-SO(I))
000019.            IF(KK)60,60,70
000020.         60 EQ=.04*Y(I)
000021.            ET=Y(I)*30/T(I)
000022.            E=EQ*ET
000023.            WE(I)=1/E
000024.            W(I)=WE(I)/WE(I)
000025.            GO TO 80
000026.         70 W(I)=1
000027.         60 CONTINUE
000028.            CALL WTDLS(W,X,Y,N,SLOPE,YINT,SDS,SDINT,SDFIT,VSUM,CORR)
000029.      C     DETERMINATION OF KM
000030.            VKM=SLOPE
000031.      C     OUTPUT
000032.            WRITE(6,10)
000033.            WRITE(6,5)SLOPE,SDS
000034.            WRITE(6,6)YINT,SDINT
000035.            WRITE(6,7)SDFIT,CORR
000036.      C     DETERMINATION OF UPPER & LOWER LIMITS
000037.            UKM=SLOPE+SDS
000038.            SKM=SLOPE-SDS
000039.            WRITE(6,8)
000040.            WRITE(6,9)VKM,UKM,SKM
000041.      C     DETERMINATION OF K3 USING KM
000042.      C     JK = 1(USING KM1;  = 2(USING KM-);= 3(USING KM+)
000043.      C     JJ = 1(USING S(I)); OR = 2(USING SO(I))
000044.            DO 500 JJ=1,2
000045.            DO 500 JK=1,3
000046.            IF(JK.EQ.1) XKM=VKM
000047.            IF(JK.EQ.2) XKM=UKM
000048.            IF(JK.EQ.3) XKM=SKM
000049.            DO 90 I=1,N
000050.            IF(JJ.EQ.1) A(I)=S(I)
000051.            IF(JJ.EQ.1) B=SO
```

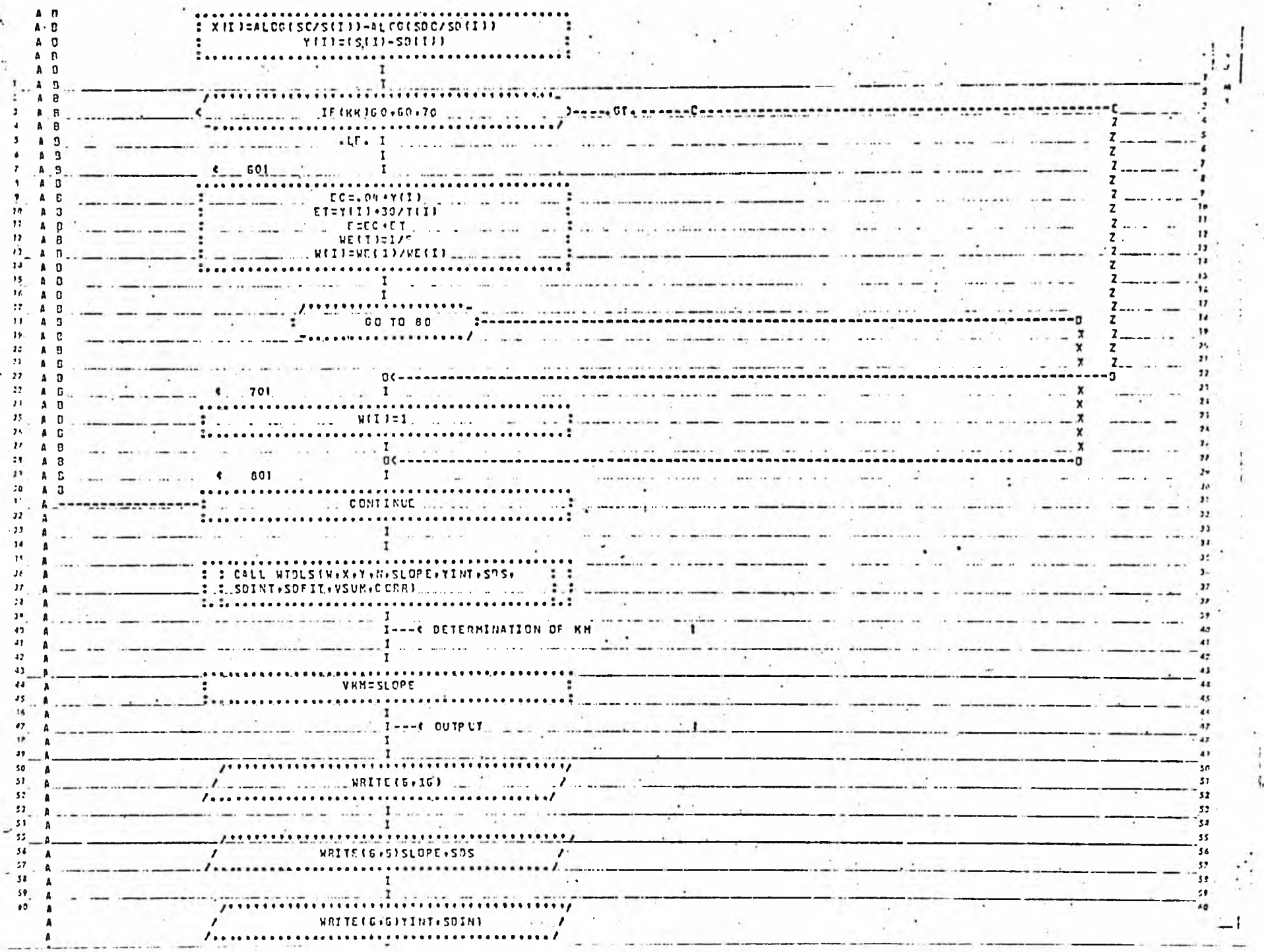
36490

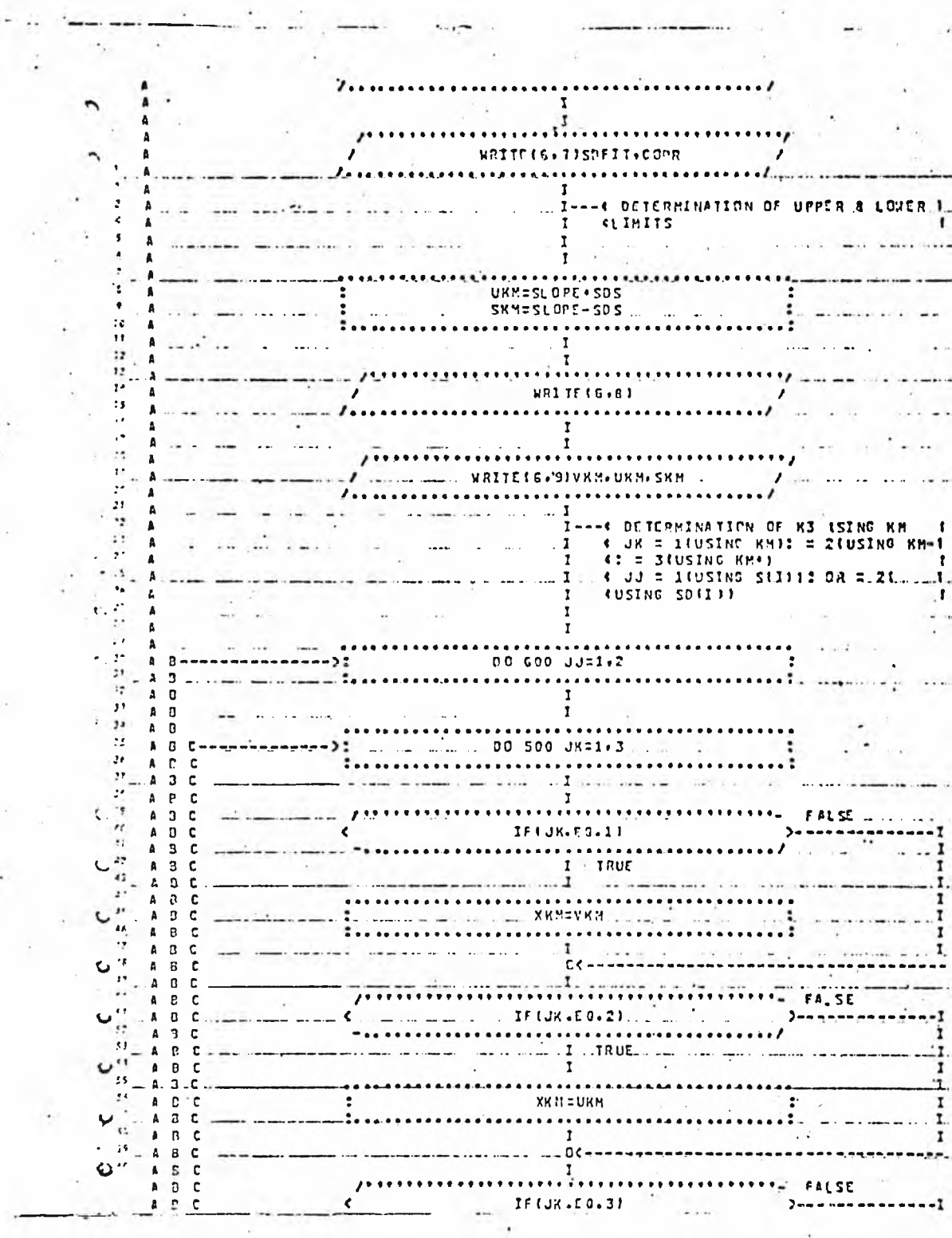


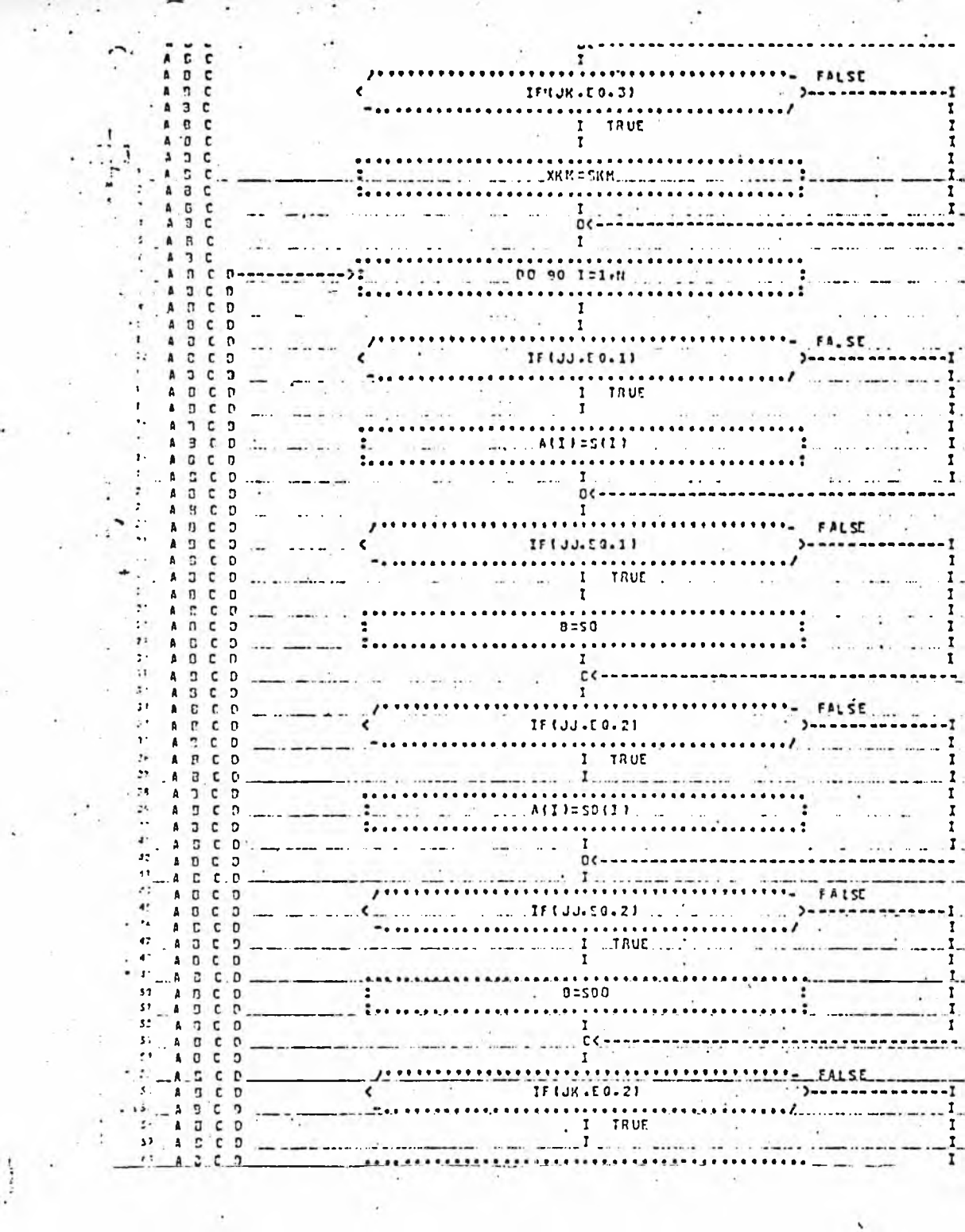
```
000052.            IF(JJ.EQ.2) A(I)=SD(I)
000053.            IF(JJ.EQ.2) B=SDO
000054.            IF(JK.EQ.2) A(I)=A(I)*1.04
000055.            IF(JK.EQ.3) A(I)=A(I)/1.04*.96
000056.            Y(I)=T(I)
000057.         90 X(I)=XKM*ALOG(B/A(I))+B-A(I)
000058.            CALL WTDLS(W,X,Y,N,SLOPE,YINT,SDS,SDINT,SDFIT,VSUM,CORR
000059.            XK3=1/(SLOPE*CONST)
000060.      C     DETERMINATION OF K3
000061.      C     OUTPUT
000062.            IF(JJ.EQ.1)WRITE(6,10)
000063.            IF(JJ.EQ.2)WRITE(6,11)
000064.            IF(JK.EQ.1)WRITE(6,12)
000065.            IF(JK.EQ.2)WRITE(6,13)
000066.            IF(JK.EQ.3)WRITE(6,14)
000067.            WRITE (6,5) SLOPE,SDS
000068.            WRITE (6,6) YINT,SDINT
000069.            WRITE (6,7) SDFIT,CORR
000070.            WRITE (6,15) XK3
000071.       500 CONTINUE
000072.       600 CONTINUE
000073.         1 FORMAT ( )
000074.         2 FORMAT (I3,2F7.5,I3)
000075.         3 FORMAT (2F8.6,F6.1)
000076.         4 FORMAT(10X,'COMPOUND NUMBER :  ',I3,//)
000077.         5 FORMAT(10X,'SLOPE',7X,': ',E13.6,6X,'STD.DEV. : ',E13.6)
000078.         6 FORMAT(10X,'Y-INTERCEPT : ',E13.6,6X,'STD.DEV. : ',E13.6
000079.         7 FORMAT(10X,'STD.ERR.EST.: ',E13.6,6X,'COR.COF. :  ',F9.6
000080.         8 FORMAT(10X,'KM-MEAN',10X,'KM-UPPER',9X,'KM-LOWER')
000081.         9 FORMAT(7X,E13.6,4X,E13.6,4X,E13.6,//)
000082.        10 FORMAT(10X,'CORRELATION USING MEAN CONC S(I)',/)
000083.        11 FORMAT(10X,'CORRELATION USING MEAN CONC SD(I)',/)
000084.        12 FORMAT(10X,'CORRELATION FOR MEAN CONC.',/)
000085.        13 FORMAT(10X,'CORRELATION FOR CONC PLUS 4%',/)
000086.        14 FORMAT(10X,'CORRELATION FOR CONC LESS 4%',/)
000087.        15 FORMAT(10X,'K3',10X,':  ',E13.6,////)
000088.        16 FORMAT(10X,'CORRELATION TO DETERMINE  KM',/)
000089.       999 CONTINUE
000090.           END
```

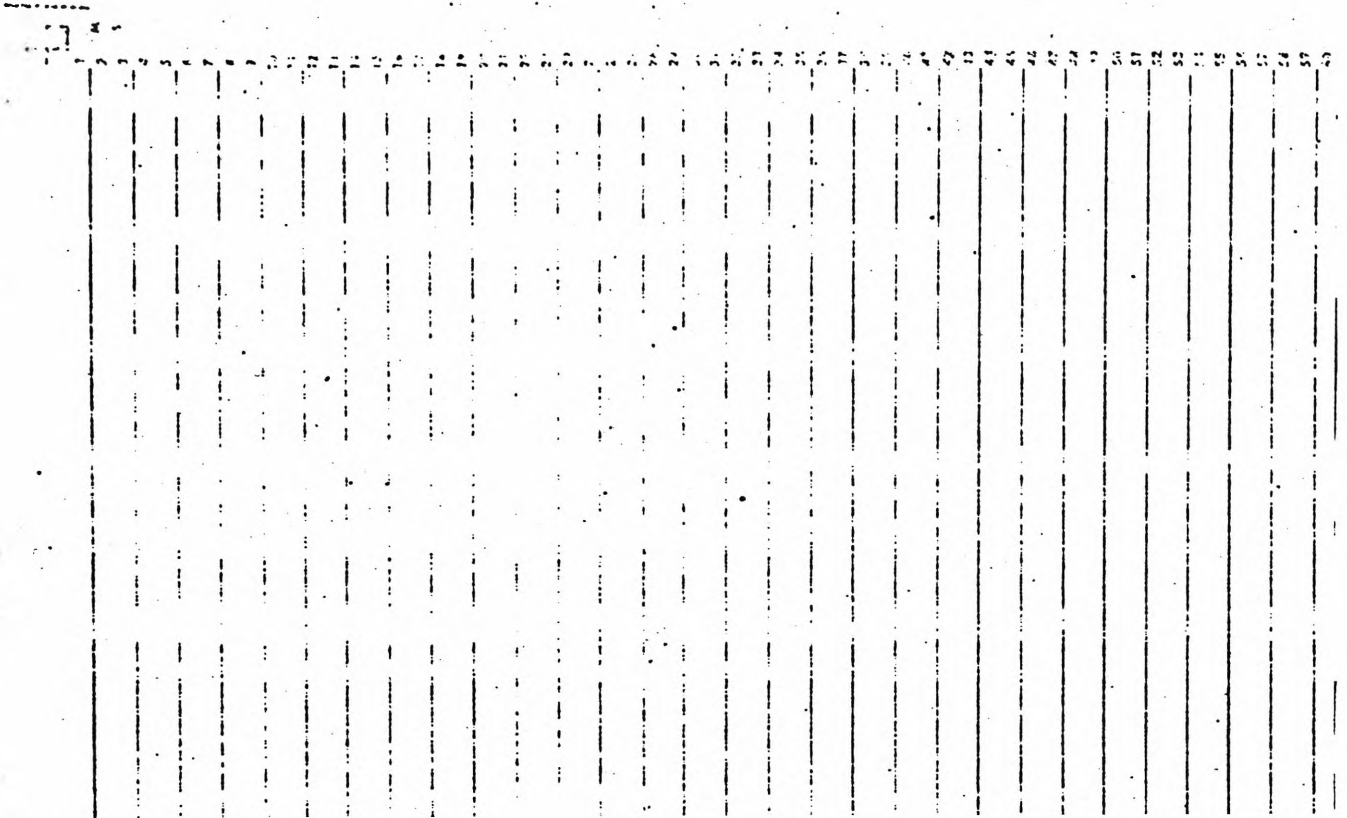

BEGIN
PROGRAM 1 : INTERGRATED MICHAELIS-MENTEN EQUATION
INPUT VARIABLES: M=NUMBER OF COMPOUNDS
N=NUMBER OF DATA POINTS
SO=INITIAL CONCENTRATION
S(I)=CONCENTRATIONS & T(I)=TIMES
KK=0 (WEIGHTED): =1 (WEIGHTED)
DIMENSION S(20),T(20),X(20),Y(20),W(20),WE(20),SO(20),A(20)
CONST=.0000002
READ(5,1)M
DO 999 J=1,M
READ(5,2) N,SO,SDO,KK
READ(5,3) (S(I),SO(I),T(I),I=1,N)
WRITE(6,4)J
DETERMINATION OF X(I),Y(I) & W(I)
50(I)
CONTINUE
DO 80 I=1,N

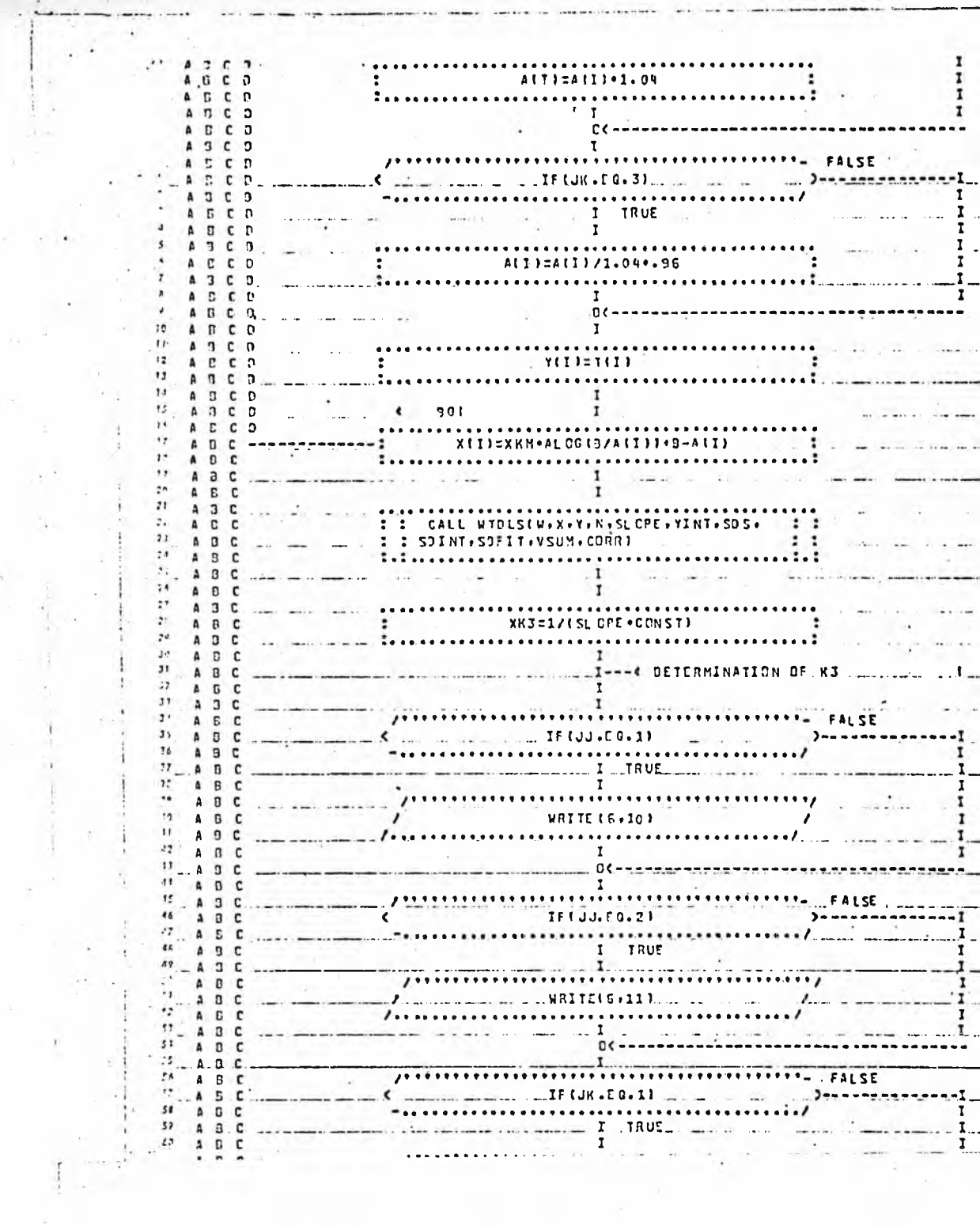


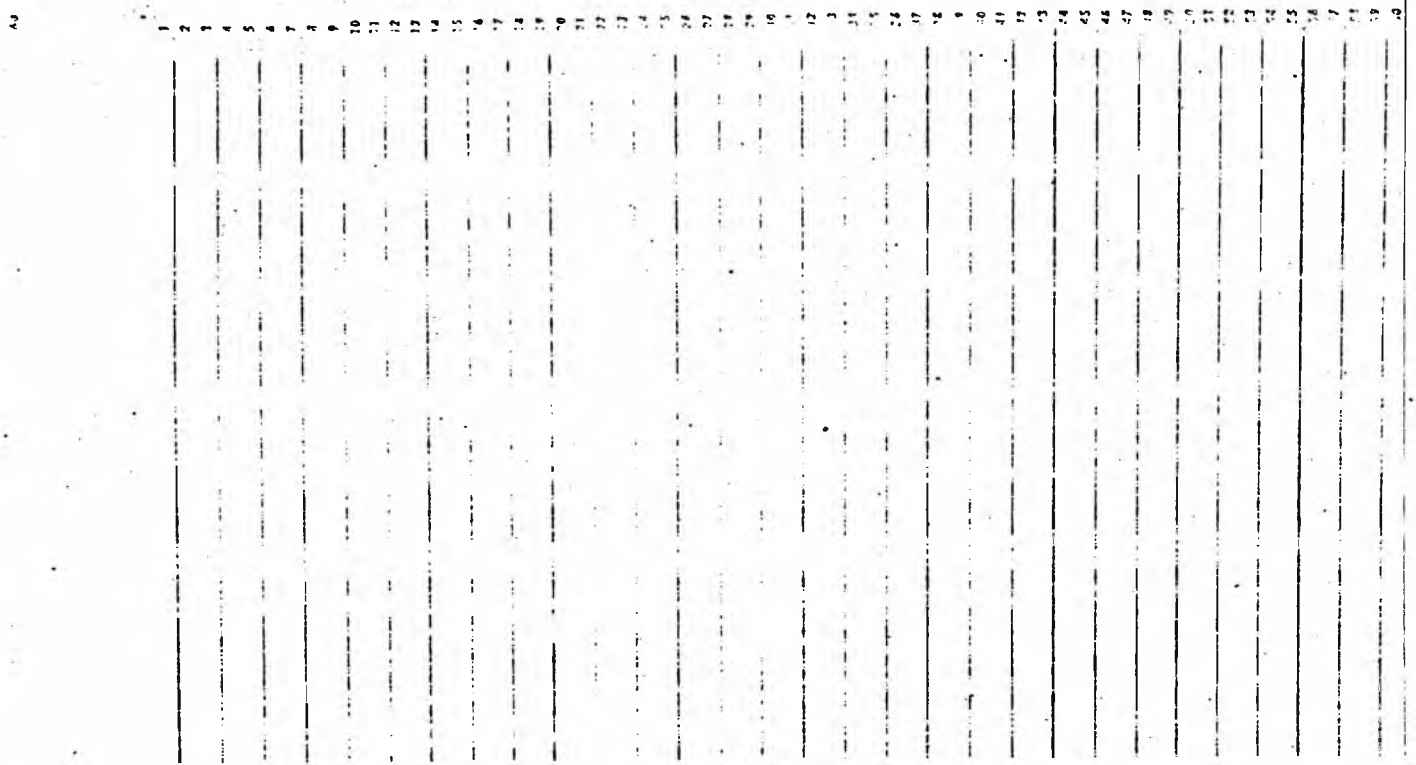

X(I)=ALOG(SC/S(I))-ALOG(SDC/SD(I))
Y(I)=S(I)-SD(I)
IF(KK)60,60,70
GT
601
EC=.04*Y(I)
ET=Y(I)*30/T(I)
E=EC+ET
WE(I)=1/E
W(I)=WE(I)/WE(I)
GO TO 80
701
W(I)=1
801
CONTINUE
CALL WTDLS(N,X,Y,N,SLOPE,YINT,SDS,
SDINT,SDFIT,VSUM,CORR)
DETERMINATION OF KM
VKM=SLOPE
OUTPUT
WRITE(6,10)
WRITE(6,5)SLOPE,SDS
WRITE(6,6)YINT,SDINT

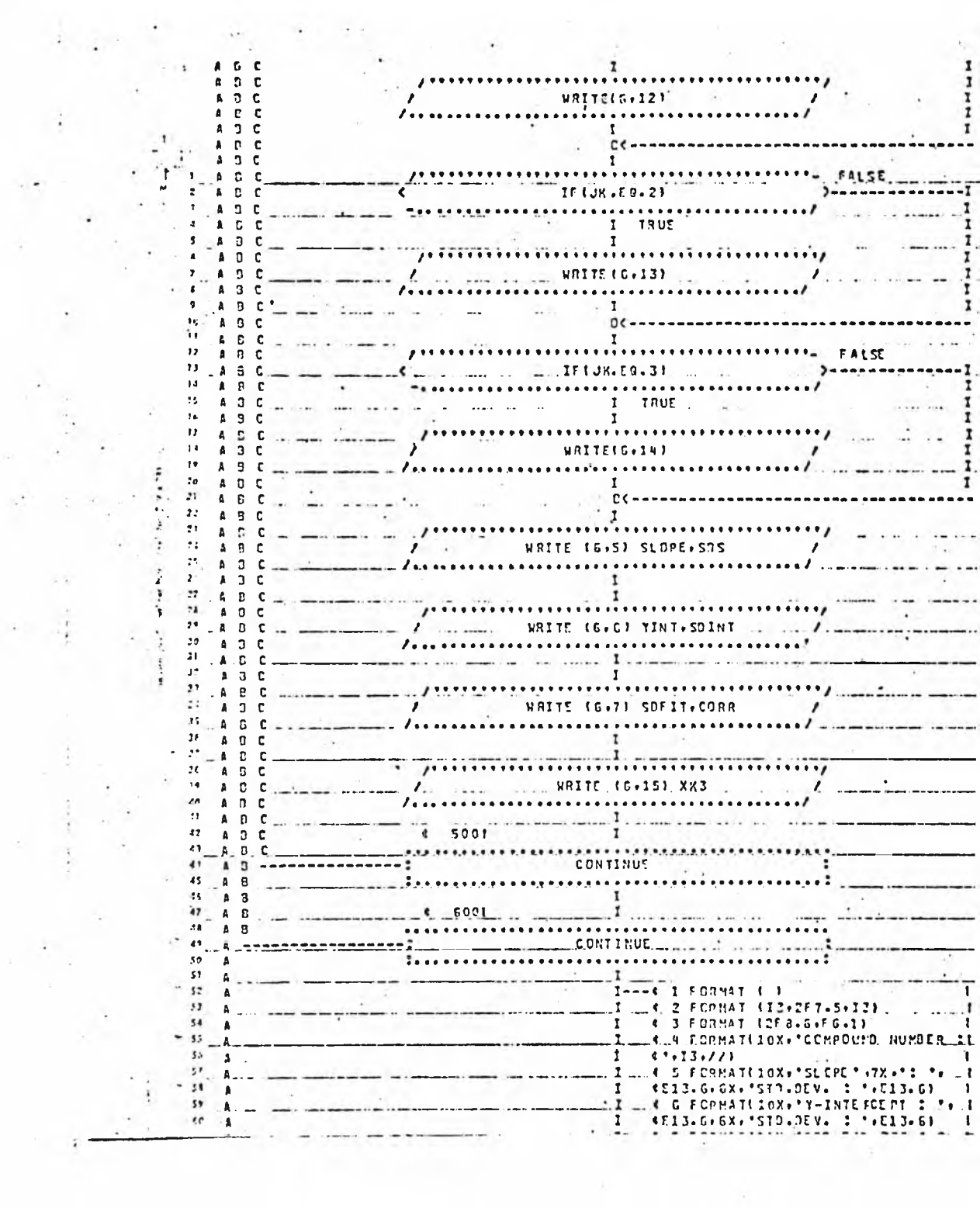

WRITE(6,7)SPEIT,COOR
DETERMINATION OF UPPER & LOWER LIMITS
UKM=SLOPE+SDS
SKM=SLOPE-SDS
WRITE(6,8)
WRITE(6,9)VKM,UKM,SKM
DETERMINATION OF K3 (SING KM
JK = 1(USING KM): = 2(USING KM-
: = 3(USING KM*)
JJ = 1(USING S(I)): OR = 2(
(USING SD(I))
DO 600 JJ=1,2
DO 500 JK=1,3
IF(JK.EQ.1)
FALSE
TRUE
XKM=VKM
IF(JK.EQ.2)
FALSE
TRUE
XKM=UKM
IF(JK.EQ.3)
FALSE


IF(JK.EQ.3)
FALSE
TRUE
XKM=SKM
DO 90 I=1,N
IF(JJ.EQ.1)
FALSE
TRUE
A(I)=S(I)
IF(JJ.EQ.1)
FALSE
TRUE
B=S0
IF(JJ.EQ.2)
FALSE
TRUE
A(I)=S0(I)
IF(JJ.EQ.2)
FALSE
TRUE
D=S00
IF(JK.EQ.2)
FALSE
TRUE




A(I)=A(I)*1.04
IF(JK.EQ.3)
FALSE
TRUE
A(I)=A(I)/1.04*.96
Y(I)=T(I)
901
X(I)=XKM*ALOG(B/A(I))+B-A(I)
CALL WTDLS(W,X,Y,N,SLOPE,YINT,SDS,
SDINT,SDFIT,VSUM,CORR)
XKJ=1/(SLOPE*CONST)
DETERMINATION OF KJ
IF(JJ.EQ.1)
FALSE
TRUE
WRITE(6,10)
IF(JJ.EQ.2)
FALSE
TRUE
WRITE(6,11)
IF(JK.EQ.1)
FALSE
TRUE




WRITE(6,12)
IF(JK.EQ.2)
FALSE
TRUE
WRITE(6,13)
IF(JK.EQ.3)
FALSE
TRUE
WRITE(6,14)
WRITE (6,5) SLOPE,SDS
WRITE (6,6) YINT,SDINT
WRITE (6,7) SDFIT,CORR
WRITE (6,15) XK3
500
CONTINUE
600
CONTINUE
1 FORMAT ( )
2 FORMAT (I2,2F7.5,I2)
3 FORMAT (2F8.6,F6.1)
4 FORMAT(10X,'COMPOUND NUMBER
'',I3,//)
5 FORMAT(10X,'SLOPE ',7X,'
6 FORMAT(10X,'Y-INTE
FORMAT(10X,'STD.DEV. : ',E13.6)
FORMAT(10X,'STD.DEV. : ',E13.6)

```
A.                                        I   $E13.6,6X,'STD.DEV. : ',E13.6)
A                                         I   $ 6 FORMAT(10X,'Y-INTERCEPT : ',
A                                         I   $E13.6,6X,'STD.DEV. : ',E13.6)
A                                         I   $ 7 FORMAT(10X,'STD.ERR.EST. : ',
A                                         I   $E13.6,6X,'COR.COF. : ',F9.6)
A                                         I   $ 8 FORMAT(10X,'KM-MEAN',10X,'KM-L
A                                         I   $OWER',9X,'KM-LOWER')
A                                         I   $ 9 FORMAT(7X,E13.6,4X,E13.6,4X,
A                                         I   $E13.6,//)
A                                         I   $ 10 FORMAT(10X,'CORRELATION
A                                         I   $USING MEAN CONC S(I)',/)
A                                         I   $ 11 FORMAT(10X,'CORRELATION
A                                         I   $USING MEAN CONC SD(I)',/)
A                                         I   $ 12 FORMAT(10X,'CORRELATION FOR
A                                         I   $MEAN CONC.',/)
A                                         I   $ 13 FORMAT(10X,'CORRELATION FOR
A                                         I   $CONC PLUS 4%',/)
A                                         I   $ 14 FORMAT(10X,'CORRELATION FOR
A                                         I   $CONC LESS 4%',/)
A                                         I   $ 15 FORMAT(10X,'K3',13X,': ',E13
A                                         I   $6,///)
A                                         I   $ 16 FORMAT(10X,'CORRELATION TO
A                                         I   $DETERMINE KM',/)
A                                         I
A                   $  9991               I
A                  .......................................
-------------------:      CONTINUE                       :
                   :.....................................:
                                          I
                                          I
                                 /.................
                                :        END        :
                                 -................./
```